PCT

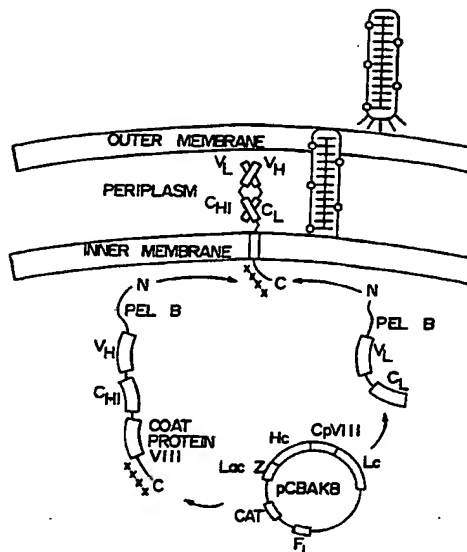## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(71) Applicant *(for all designated States except US)*: THE SCRIPPS RESEARCH INSTITUTE [US/US]; 10666 North Torrey Pines Road, La Jolla, CA 92037 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : KANG, Angray [US/US]; 7706 Hillside Drive, La Jolla, CA 92037 (US). BARBAS, Carlos [US/US]; 3131-H Via Alicante, La Jolla, CA 92037 (US). LERNER, Richard, A. [US/US]; 7750 Roseland Drive, East, La Jolla, CA 92037 (US).

(74) Agents: FITTING, Thomas et al.; The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037 (US).

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(54) Title: HETERODIMERIC RECEPTOR LIBRARIES USING PHAGEMIDS

(57) Abstract

Filamentous phage comprising a matrix of cpVIII proteins encapsulating a genome encoding first and second polypeptides of an antogenously assembling receptor, such as an antibody, and a receptor comprised of the first and second polypeptides surface-integrated into the matrix via a filamentous phage coat protein membrane anchor domain fused to at least one of the polypeptides.

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HETERODIMERIC RECEPTOR LIBRARIES USING PHAGEMIDS

Technical Field

The present invention relates to cloning vectors
and methods for producing a library of DNA molecules
5   capable of expressing a fusion polypeptide on the
surface of a filamentous phage particle.

Background

10   Filamentous bacteriophages are a group of related
viruses that infect bacteria. They are termed
filamentous because they are long and thin particles
comprised of an elongated capsule that envelopes the
deoxyribonucleic acid (DNA) that forms the
15   bacteriophage genome. The F pili filamentous
bacteriophage (Ff phage) infect only gram-negative
bacteria by specifically adsorbing to the tip of F
pili, and include fd, f1 and M13.
The mature capsule of Ff phage is comprised of a
20   coat of five phage-encoded gene products: cpVIII, the
major coat protein product of gene VIII that forms the
bulk of the capsule; and four minor coat proteins,
cpIII and cpIV at one end of the capsule and cpVII and
cpIX at the other end of the capsule. The length of
25   the capsule is formed by 2500 to 3000 copies of cpVIII
in an ordered helix array that forms the
characteristic filament structure. About five copies
each of the minor coat proteins are present at the
ends of the capsule. The gene III-encoded protein
30   (cpIII) is typically present in 4 to 6 copies at one
end of the capsule and serves as the receptor for
binding of the phage to its bacterial host in the
initial phase of infection. For detailed reviews of
Ff phage structure, see Rasched et al., _Microbiol._
35   _Rev._, 50:401-427 (1986); and Model et al., in "The

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Bacteriophages, Volume 2", R. Calendar, Ed., Plenum
Press, pp. 375-456 (1988).

The assembly of a Ff phage particle involves
highly complex mechanics. No phage particles are
assembled within a host cell; rather, they are
assembled during extrusion of the viral genome through
the host cell's membrane. Prior to extrusion, the
major coat protein cpVIII and the minor coat protein
cpIII are synthesized and transported to the host
cell's membrane. Both cpVIII and cpIII are anchored
in the host cell membrane prior to their incorporation
into the mature particle. In addition, the viral
genome is produced and coated with cpV protein.
During the extrusion process, cpV-coated genomic DNA
is stripped of the cpV coat and simultaneously re-
coated with the mature coat proteins. The assembly
mechanisms that control transferral of these proteins
from the membrane to the particle is not presently
known.

Both cpIII and cpVIII proteins include two
domains that provide signals for assembly of the
mature phage particle. The first domain is a
secretion signal that directs the newly synthesized
protein to the host cell membrane. The secretion
signal is located at the amino terminus of the
polypeptide and targets the polypeptide at least to
the cell membrane. The second domain is a membrane
anchor domain that provides signals for association
with the host cell membrane and for association with
the phage particle during assembly. This second signal
for both cpVIII and cpIII comprises at least a
hydrophobic region for spanning the membrane.

cpVIII has been extensively studied as a model
membrane protein because it can integrate into lipid
bilayers such as the cell membrane in an asymmetric

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orientation with the acidic amino terminus toward the outside and the basic carboxy terminus toward the inside of the membrane. The mature protein is about 50 amino acid residues in length of which 11 residues

5   provide the carboxy terminus, 19 residues provide the hydrophobic transmembrane region, and the remaining residues comprise the amino terminus. Considerable research has been done on the secretion signal region of cpVIII to advance the study of membrane protein

10   synthesis and targeting to membranes. However, little is known about the changes that are tolerated in the structure of the cpVIII membrane anchor region that would allow for assembly of phage particles.

Manipulation of the sequence of cpIII shows that

15   the C-terminal 23 amino acid residue stretch of hydrophobic amino acids normally responsible for a membrane anchor function can be altered in a variety of ways and retain the capacity to associate with membranes. However, those anchor-modified cpIII

20   proteins lost their ability to genetically complement gene III mutants indicating that the requirements of a membrane anchor for functional assembly have not been elucidated.

Ff phage-based expression vectors have been

25   described in which the entire cpIII amino acid residue sequence was modified by insertion of short polypeptide "epitopes" [Parmely et al., Gene, 73:305-318 (1988); and Cwirla et al., Proc. Natl. Acad. Sci. USA, 87:6378-6382 (1990)] or an amino acid residue

30   sequence defining a single chain antibody domain. McCafferty et al., Science, 348:552-554 (1990). These hybrid proteins were synthesized and assembled onto phage particles in amounts of about 5 copies per particle, a density at which normal cpIII is usually

35   found. However, these expressed fusion proteins

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include the entire cpIII amino acid residue sequence
and do not suggest fusion proteins that utilize only
the carboxy terminal membrane anchor domain of cpIII.

In addition, no expression system has been
5   described in which a phage coat protein has been
engineered to allow assembly of a heterodimeric
molecule that is functional and capable of
incorporation into the coat of a phage particle.

10   Brief Summary of the Invention

A new surface-integration technology has been
discovered for expressing a heterodimeric recombinant
gene product on the surface of a filamentous phage
containing the recombinant gene. The invention uses a
15   filamentous phage coat protein membrane anchor domain
as a means for linking gene-product and gene during
the assembly stage of filamentous phage replication.

That is, during filamentous phage replication,
coat proteins assemble into a matrix which
20   encapsulates the phage genome. It has now been
discovered that (1) phage assembly is not disrupted
when recombinant filamentous phage coat proteins are
present, (2) recombinant filamentous phage coat
proteins can be integrated into the assembling matrix,
25   and (3) integration into the matrix can be directed to
occur in a surface-accessible orientation.

The present invention can be advantageously
applied to the production of heterodimeric receptors
of predetermined specificity, i.e., it can be used to
30   produce antibodies, T-cell receptors and the like that
bind a preselected ligand.

Thus, the present invention provides for linking
the functions of heterodimeric receptor recognition
and filamentous phage replication in a method for
35   isolating a heterodimeric receptor and the gene that

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encodes receptor. The method produces a filamentous
phage comprised of a matrix of gene VIII-encoded
proteins that encapsulate a recombinant genome. The
recombinant genome contains genes encoding the
heterodimeric receptor polypeptides. The
heterodimeric receptor is surface-integrated into the
encapsulating matrix via a filamentous phage coat
protein's membrane anchor domain that is fused by a
peptide bond during translation to one of the
heterodimeric receptor polypeptides. The
heterodimeric receptor polypeptides and the genes
which encode the polypeptides are physically linked
during the assembly stage of the phage replication
cycle. Specifically binding the receptor-coated phage
to a solid-support advantageously provides a means for
isolating a recombinant genome that encodes a desired
heterodimeric receptor from a diverse library of
recombinant genomes.

In one embodiment, the present invention
contemplates an antibody molecule comprising heavy-
and light-chain polypeptides, said heavy-chain
polypeptide comprising a V_H-domain flanked by an
amino-terminal prokaryotic secretion signal domain and
a carboxy-terminal filamentous phage membrane anchor
domain, said light chain polypeptide comprising a V_L-
domain fused to an amino-terminal prokaryotic
secretion signal domain.

In another embodiment, the present invention
contemplates a vector for expressing a fusion
polypeptide, said vector comprising upstream and
downstream translatable DNA sequences operatively
linked via a sequence of nucleotides adapted for
directional ligation of an insert DNA, said upstream
sequence encoding a prokaryotic secretion signal, said
downstream sequence encoding a filamentous phage

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membrane anchor, said translatable DNA sequences
operatively linked to a set of DNA expression signals
for expression of said translatable DNA sequences as
portions of said fusion polypeptide.

Brief Description of the Drawings
        In the drawings forming a portion of this
disclosure:
        Figure 1 illustrates a schematic diagram of the
immunoglobulin molecule showing the principal
structural features. The circled area on the heavy
chain represents the variable region (V_H), a
polypeptide containing a biologically active (ligand
binding) portion of that region, and a gene coding for
that polypeptide, are produced by the methods of the
present invention.
        Figure 2A is a diagrammatic sketch of a heavy (H)
chain of human IgG (IgG1 subclass). Numbering is from
the N-terminus on the left to the C-terminus on the
right. Note the presence of four domains, each
containing an intrachain disulfide bond (S-S) spanning
approximately 60 amino acid residues. The symbol CHO
stands for carbohydrate. The V region of the heavy
(H) chain (V_H) resembles V_L in having three
hypervariable CDR (not shown).
        Figure 2B is a diagrammatic sketch of a human
light (Kappa) chain (Panel 1). Numbering is from the
N-terminus on the left to the C-terminus on the right.
Note the intrachain disulfide bond (S-S) spanning
about the same number of amino acid residues in the V_L
and C_L domains. Panel 2 shows the locations of the
complementarity-determining regions (CDR) in the V_L
domain. Segments outside the CDR are the framework
segments (FR).
        Figure 3 illustrates the sequence of the double-

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stranded synthetic DNA inserted into Lambda Zap to
produce a Lambda Hc2 expression vector. The
preparation of the double-stranded synthetic DNA
insert is described in Example 1a(ii). The various
features required for this vector to express the V_H-
coding DNA homologs include the Shine-Dalgarno
ribosome binding site, a leader sequence to direct the
expressed protein to the periplasm as described by
Mouva et al., <u>J. Biol. Chem.</u>, 255:27, 1980, and
various restriction enzyme sites used to operatively
link the V_H homologs to the expression vector. The V_H
expression vector sequence also contains a short
nucleic acid sequence that codes for amino acids
typically found in variable regions heavy chain (V_H
backbone). This V_H backbone is just upstream and in
the proper reading as the V_H DNA homologs that are
operatively linked into the Xho I and Spe I cloning
sites. The sequences of the top and bottom strands of
the double-stranded synthetic DNA insert are listed
respectively as SEQ ID NO 1 and SEQ ID NO 2. The
synthetic DNA insert is directionally ligated into
Lambda Zap II digested with the restriction enzymes
Not 1 and Xho I to form Lambda Hc2 expression vector.

Figure 4 illustrates the major features of the
bacterial expression vector Lambda Hc2 (V_H expression
vector). The synthetic DNA sequence from Figure 3 is
shown at the top along with the T_3 polymerase promoter
from Lambda Zap II. The orientation of the insert in
Lambda Zap II is shown. The V_H DNA homologs are
inserted into the Xho I and Spe I cloning sites. The
read through transcription produces the decapeptide
epitope (tag) that is located just 3' of the cloning
site.

Figure 5 illustrates the sequence of the double-
stranded synthetic DNA inserted into Lambda Zap to

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produce a Lambda Lc2 expression vector. The various
features required for this vector to express the V_L-
coding DNA homologs are described in Figure 3. The
V_L-coding DNA homologs are operatively linked into the
5   Lc2 sequence at the Sac I and Xho I restriction sites.
The sequences of the top and bottom strands of the
double-stranded synthetic DNA insert are listed
respectively as SEQ ID NO 3 and SEQ ID NO 4. The
synthetic DNA insert is directionally ligated into
10   Lambda Zap II digested with the restriction enzymes
Sac I and Not I to form Lambda Lc2 expression vector.
       Figure 6 illustrates the major features of the
bacterial expression vector Lc2 (V_L expression
vector). The synthetic DNA sequence from Figure 5 is
15   shown at the top along with the T_3 polymerase promoter
from Lambda Zap II. The orientation of the insert in
Lambda Zap II is shown. The V_L DNA homologs are
inserted into the Sac I and Xho I cloning sites.
       Figure 7 illustrates the dicistronic expression
20   vector, pComb, in the form of a phagemid expression
vector. To produce pComb, phagemids were first
excised from the expression vectors, Lambda Hc2 and
Lambda Lc2, using an _in vivo_ excision protocol
according to manufacturers instructions (Stratagene,
25   La Jolla, California). The pComb expression vector is
prepared from Lambda Hc2 and Lambda Lc2 which do not
contain V_H-coding or V_L-coding DNA homologs. The _in
vivo_ excision protocol moved the cloned insert from
the Lambda Hc2 and Lc2 vectors into a phagemid vector.
30   The resultant phagemids contained the same nucleotide
sequences for antibody fragment cloning and expression
as did the parent vectors. Hc2 and Lc2 phagemid
expression vectors were separately restriction
digested with Sca I and EcoR I. The linearized
35   phagemids were ligated via the Sca I and EcoR I

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cohesive termini to form the dicistronic
(combinatorial) vector, pComb.

Figure 8 illustrates a schematic diagram of the
composition of pCBAK8-2b phagemid vector, the pathway
for Fab assembly and incorporation in phage coat. The
vector carries the chloramphenicol acetyl transferase
(CAT) marker gene in addition to the nucleotide
residue sequences encoding the Fd-cpVIII fusion
polypeptide and the kappa chain. The f1 phage origin
of replication facilitates the generation of single
stranded phagemid. The isopropyl
thiogalactopyranoside (IPTG) induced expression of a
dicistronic message encoding the Fd-cpVIII fusion (V_H,
C_{H1}, cpVIII) and the light chain (V_L, C_L) leads to the
formation of heavy and light chains. Each chain is
delivered to the periplasmic space by the pelB target
sequence, which is subsequently cleaved. The heavy
chain is anchored in the membrane by cpVIII fusion
while the light chain is secreted into the periplasm.
The heavy chain in the presence of light chain
assembles to form Fab molecules. The Fabs are
incorporated into phage particles via cpVIII (black
dots).

Figure 9 illustrates the electron micrographic
localization of 5-7 nm colloidal gold particles coated
with NPN-BSA conjugate along the surface of
filamentous phage, and from phage emerging from a
bacterial cell. Panel 9A shows filamentous phage
emerging from the surface of the bacterial cell
specifically labelled with the colloidal gold
particles coated with BSA-NPN antigen. Panel 9B shows
a portion of a mature filamentous phage on the length
of which is exhibited the labelling of antigen binding
sites.

Figure 10 illustrates the results of a two-site

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ELISA for assaying for the presence and function of
Fab antibody attached to the surface of bacteriophage
particles as described in Example 4b. For expression
of Fab antibody on phage surfaces, XL1-Blue cells were
5   transformed with the phagemid expression vector,
pCBAK8-2b. The inducer, isopropyl
thiogalactopyranoside (IPTG), was admixed with the
bacterial suspension at a final concentration of 1 mM
for one hour. Helper phage was then admixed with the
10   bacterial suspension to initiate the generation of
copies of the sense strand of the phagemid DNA. After
a two hour maintenance period, bacterial supernatants
containing bacteriophage particles were collected for
assaying in ELISA.
15   Specific titratable binding of NPN-Fab-expressing
bacteriophage particles to NPN-coated plates was
exhibited. No binding was detected with helper phage
alone.
           Figure 11 illustrates the inhibition of NPN-Fab
20   expressing bacteriophage to NPN antigen-coated plates
with the addition of increasing amounts of free
hapten. The assays were performed as described in
Figure 10. Complete inhibition of binding was
observed with 5 ng of added free NPN hapten.
25   Figure 12 illustrates schematically the process
of mutagenizing the CDR3 region of a heavy chain
fragment resulting in an alteration of binding
specificity. The oligonucleotide primers are
indicated by black bars. The process is described in
30   Example 6.
           Figure 13 illustrates the amino acid sequences
and corresponding SEQ ID NO of the heavy chain of the
CDR3 region of the starting and selected clones, and
the affinities of the clones for free fluorescein and
35   FI-BSA. The asterisk indicates the approximate Kd as

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determined by competitive ELISA.

Figure 14 illustrates the amino acid sequences
and corresponding SEQ ID NO of the light chain of the
CDR3 region of the starting and selected clones, and
5   the affinities of the clones for free fluorescein and
FI-BSA. The asterisk indicates the approximate Kd as
determined by competitive ELISA.

Detailed Description of the Invention

10   A.   Definitions

Amino Acid Residue: An amino acid formed
upon chemical digestion (hydrolysis) of a polypeptide
at its peptide linkages. The amino acid residues
described herein are preferably in the "L" isomeric

15   form. However, residues in the "D" isomeric form can
be substituted for any L-amino acid residue, as long
as the desired functional property is retained by the
polypeptide. NH_2 refers to the free amino group
present at the amino terminus of a polypeptide. COOH

20   refers to the free carboxy group present at the
carboxy terminus of a polypeptide. In keeping with
standard polypeptide nomenclature (described in J.
Biol. Chem., 243:3552-59 (1969) and adopted at 37
C.F.R. 1.822(b)(2)), abbreviations for amino acid

25   residues are shown in the following Table of
Correspondence:

TABLE OF CORRESPONDENCE

	SYMBOL		AMINO ACID
30	1-Letter	3-Letter	
	Y	Tyr	tyrosine
	G	Gly	glycine
	F	Phe	phenylalanine
	M	Met	methionine
35	A	Ala	alanine

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S	Ser	serine
I	Ile	isoleucine
L	Leu	leucine
T	Thr	threonine
V	Val	valine
P	Pro	proline
K	Lys	lysine
H	His	histidine
Q	Gln	glutamine
E	Glu	glutamic acid
Z	Glx	Glu and/or Gln
W	Trp	tryptophan
R	Arg	arginine
D	Asp	aspartic acid
N	Asn	asparagine
B	Asx	Asn and/or Asp
C	Cys	cysteine
J	Xaa	Unknown or other

It should be noted that all amino acid residue
sequences represented herein by formulae have a left-
to-right orientation in the conventional direction of
amino terminus to carboxy terminus. In addition, the
phrase "amino acid residue" is broadly defined to
include the amino acids listed in the Table of
Correspondence and modified and unusual amino acids,
such as those listed in 37 CFR 1.822(b)(4), and
incorporated herein by reference. Furthermore, it
should be noted that a dash at the beginning or end of
an amino acid residue sequence indicates a peptide
bond to a further sequence of one or more amino acid
residues or a covalent bond to an amino-terminal group
such as NH_2 or acetyl or to a carboxy-terminal group
such as COOH.

Nucleotide: A monomeric unit of DNA or RNA

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consisting of a sugar moiety (pentose), a phosphate, and a nitrogenous heterocyclic base. The base is linked to the sugar moiety via the glycosidic carbon (1' carbon of the pentose) and that combination of
5   base and sugar is a nucleoside. When the nucleoside contains a phosphate group bonded to the 3' or 5' position of the pentose it is referred to as a nucleotide. A sequence of operatively linked nucleotides is typically referred to herein as a "base
10 sequence" or "nucleotide sequence", and their grammatical equivalents, and is represented herein by a formula whose left to right orientation is in the conventional direction of 5'-terminus to 3'-terminus.
        Base Pair (bp): A partnership of adenine (A)
15 with thymine (T), or of cytosine (C) with guanine (G) in a double stranded DNA molecule. In RNA, uracil (U) is substituted for thymine.
        Nucleic Acid: A polymer of nucleotides, either single or double stranded.
20   Polynucleotide: a polymer of single or double stranded nucleotides. As used herein "polynucleotide" and its grammatical equivalents will include the full range of nucleic acids. A polynucleotide will typically refer to a nucleic acid molecule comprised
25 of a linear strand of two or more deoxyribonucleotides and/or ribonucleotides. The exact size will depend on many factors, which in turn depends on the ultimate conditions of use, as is well known in the art. The polynucleotides of the present invention include
30 primers, probes, RNA/DNA segments, oligonucleotides or "oligos" (relatively short polynucleotides), genes, vectors, plasmids, and the like.
        Gene: A nucleic acid whose nucleotide sequence codes for an RNA or polypeptide. A gene can be either
35 RNA or DNA.

Duplex DNA: a double-stranded nucleic acid molecule comprising two strands of substantially complementary polynucleotides held together by one or more hydrogen bonds between each of the complementary bases present in a base pair of the duplex. Because the nucleotides that form a base pair can be either a ribonucleotide base or a deoxyribonucleotide base, the phrase "duplex DNA" refers to either a DNA-DNA duplex comprising two DNA strands (ds DNA), or an RNA-DNA duplex comprising one DNA and one RNA strand.

Complementary Bases: Nucleotides that normally pair up when DNA or RNA adopts a double stranded configuration.

Complementary Nucleotide Sequence: A sequence of nucleotides in a single-stranded molecule of DNA or RNA that is sufficiently complementary to that on another single strand to specifically hybridize to it with consequent hydrogen bonding.

Conserved: A nucleotide sequence is conserved with respect to a preselected (reference) sequence if it non-randomly hybridizes to an exact complement of the preselected sequence.

Hybridization: The pairing of substantially complementary nucleotide sequences (strands of nucleic acid) to form a duplex or heteroduplex by the establishment of hydrogen bonds between complementary base pairs. It is a specific, i.e. non-random, interaction between two complementary polynucleotides that can be competitively inhibited.

Nucleotide Analog: A purine or pyrimidine nucleotide that differs structurally from A, T, G, C, or U, but is sufficiently similar to substitute for the normal nucleotide in a nucleic acid molecule.

DNA Homolog: Is a nucleic acid having a preselected conserved nucleotide sequence and a

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sequence coding for a receptor capable of binding a
preselected ligand.

Recombinant DNA (rDNA) molecule: a DNA molecule
produced by operatively linking two DNA segments.
5   Thus, a recombinant DNA molecule is a hybrid DNA
molecule comprising at least two nucleotide sequences
not normally found together in nature. rDNA's not
having a common biological origin, i.e.,
evolutionarily different, are said to be
10   "heterologous".

Vector: a rDNA molecule capable of autonomous
replication in a cell and to which a DNA segment,
e.g., gene or polynucleotide, can be operatively
linked so as to bring about replication of the
15   attached segment. Vectors capable of directing the
expression of genes encoding for one or more
polypeptides are referred to herein as "expression
vectors". Particularly important vectors allow
cloning of cDNA (complementary DNA) from mRNAs
20   produced using reverse transcriptase.

Receptor: A receptor is a molecule, such as a
protein, glycoprotein and the like, that can
specifically (non-randomly) bind to another molecule.

Antibody: The term antibody in its various
25   grammatical forms is used herein to refer to
immunoglobulin molecules and immunologically active
portions of immunoglobulin molecules, i.e., molecules
that contain an antibody combining site or paratope.
Exemplary antibody molecules are intact immunoglobulin
30   molecules, substantially intact immunoglobulin
molecules and portions of an immunoglobulin molecule,
including those portions known in the art as Fab,
Fab', F(ab')$_2$ and F(v).

Antibody Combining Site: An antibody combining
35   site is that structural portion of an antibody

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molecule comprised of a heavy and light chain variable
and hypervariable regions that specifically binds
(immunoreacts with) an antigen. The term immunoreact
in its various forms means specific binding between an
5   antigenic determinant-containing molecule and a
molecule containing an antibody combining site such as
a whole antibody molecule or a portion thereof.

Monoclonal Antibody: The phrase monoclonal
antibody in its various grammatical forms refers to a
10   population of antibody molecules that contains only
one species of antibody combining site capable of
immunoreacting with a particular antigen. A
monoclonal antibody thus typically displays a single
binding affinity for any antigen with which it
15   immunoreacts. A monoclonal antibody may therefore
contain an antibody molecule having a plurality of
antibody combining sites, each immunospecific for a
different antigen, e.g., a bispecific monoclonal
antibody.

20   Fusion Polypeptide: A polypeptide comprised of at
least two polypeptides and a linking sequence to
operatively link the two polypeptides into one
continuous polypeptide. The two polypeptides linked
in a fusion polypeptide are typically derived from two
25   independent sources, and therefore a fusion
polypeptide comprises two linked polypeptides not
normally found linked in nature.

Upstream: In the direction opposite to the
direction of DNA transcription, and therefore going
30   from 5' to 3' on the non-coding strand, or 3' to 5' on
the mRNA.

Downstream: Further along a DNA sequence in the
direction of sequence transcription or read out, that
is traveling in a 3'- to 5'-direction along the non-
35   coding strand of the DNA or 5'- to 3'-direction along

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the RNA transcript.

Cistron: Sequence of nucleotides in a DNA
molecule coding for an amino acid residue sequence and
including upstream and downstream DNA expression
control elements.

Stop Codon: Any of three codons that do not code
for an amino acid, but instead cause termination of
protein synthesis. They are UAG, UAA and UGA and are
also referred to as a nonsense or termination codon.

Leader Polypeptide: A short length of amino acid
sequence at the amino end of a polypeptide, which
carries or directs the polypeptide through the inner
membrane and so ensures its eventual secretion into
the periplasmic space and perhaps beyond. The leader
sequence peptide is commonly removed before the
polypeptide becomes active.

Reading Frame: Particular sequence of contiguous
nucleotide triplets (codons) employed in translation.
The reading frame depends on the location of the
translation initiation codon.

B.   Filamentous Phage

The present invention contemplates a
filamentous phage comprising a matrix of proteins
encapsulating a genome encoding first and second
polypeptides capable of forming a heterodimeric
receptor. The phage further contains a heterodimeric
receptor comprised of the first and second
polypeptides surface-integrated into the matrix via a
filamentous phage membrane anchor domain fused to at
least one of the first or second polypeptides. The
heterodimeric receptor has the capacity to bind ligand
and therefor is referred to as a ligand-binding
heterodimeric receptor.

The heterodimeric receptor in a preferred

embodiment is an epitope-binding complex. That is, a complex of first and second polypeptides capable of binding an epitope. Preferably, the first and second polypeptides are antibody heavy chain and light chain

5   polypeptides.

The first and second polypeptides are ·capable of autogenous assembly into a functional epitope-binding complex (heterodimeric receptor), which is then expressed on the outer surface of the phage in a

10   manner accessible to ligand, i.e., they are surface-integrated into the phage. Thus, an epitope-binding complex is typically present on the surface of a phage of this invention. Typically, the epitope-binding complex is comprised of a linking polypeptide that

15   contains a filamentous phage membrane anchor domain, such as a polypeptide described in Section C, and a non-linking polypeptide(s). Preferred are phage having a cpIII or cpVIII membrane anchor domain fused to a polypeptide of the heterodimeric complex as

20   described further herein.

Because the receptor is linked to the phage in a surface accessible manner, the phage can be advantageously used as a solid-phase affinity sorbent. In preferred embodiments, the phage are linked,

25   preferably removably linked, to a solid (aqueous insoluble) matrix such as agarose, cellulose, synthetic reins, polysaccharides and the like. For example, transformants shedding the phage can be applied to and retained in a column and maintained

30   under conditions that support shedding of the phage. An aqueous composition containing a ligand that binds to the receptor expressed by the phage is then passed through the column at a predetermined rate and under receptor-binding conditions to form a solid-phase

35   receptor-ligand complex. The column is then washed to

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remove unbound material, leaving the ligand bound to
the solid-phase phage. The ligand can then be removed
and recovered by washing the column with a buffer that
promotes dissociation of the receptor-ligand complex.

5   Alternatively, purified phage can be admixed with
a aqueous solution containing the ligand to be
affinity purified. The receptor/ligand binding
reaction admixture thus formed is maintained for a
time period and under binding conditions sufficient

10   for a phage-linked receptor-ligand complex to form.
The phage-bound ligand (ligand-bearing phage) are then
separated and recovered from the unbound materials,
such as by centrifugation, electrophoresis,
precipitation, and the like.

15   Phage of this invention can be labeled when used
in a diagnostic method of this invention. Preferred
labels include radioactively labeled nucleic acids
incorporated into the phage genome, or radioactively
labeled amino acids incorporated into protein

20   components of the phage particle. Preparation of
labeled phage can be routinely prepared by growing
phage as described herein, but including radiolabeled
nucleotides or radiolabeled amino acids in the culture
medium for incorporation into nucleic acids or

25   polypeptides of the phage, respectively. Exemplary
labels are ^{3}H-thymidine or ^{35}S-methionine. Other
isotopic labels and other nucleotide or amino acid
precursors are readily available to one skilled in the
art. The labeled phage preferably contains sufficient

30   label to be detectable in a ligand binding assay of
this invention, i.e., the phage is detectably labeled.

C.   DNA Expression Vectors
            A vector of the present invention is a
35   recombinant DNA (rDNA) molecule adapted for receiving

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and expressing translatable DNA sequences in the form
of a fusion polypeptide containing a filamentous phage
membrane anchor domain and a prokaryotic secretion
signal domain. The vector comprises a cassette that
5   includes upstream and downstream translatable DNA
sequences operatively linked via a sequence of
nucleotides adapted for directional ligation to an
insert DNA. The upstream translatable sequence
encodes the secretion signal as defined herein. The
10   downstream translatable sequence encodes the
filamentous phage membrane anchor as defined herein.
The cassette preferably includes DNA expression
control sequences for expressing the fusion
polypeptide that is produced when an insert
15   translatable DNA sequence (insert DNA) is
directionally inserted into the cassette via the
sequence of nucleotides adapted for directional
ligation. The filamentous phage membrane anchor is
preferably a domain of the cpIII or cpVIII coat
20   protein capable of binding the matrix of a filamentous
phage particle, thereby incorporating the fusion
polypeptide onto the phage surface.

            An expression vector is characterized as being
capable of expressing, in a compatible host, a
25   structural gene product such as a fusion polypeptide
of the present invention.

            As used herein, the term "vector" refers to a
nucleic acid molecule capable of transporting between
different genetic environments another nucleic acid to
30   which it has been operatively linked. Preferred
vectors are those capable of autonomous replication
and expression of structural gene products present in
the DNA segments to which they are operatively linked.

            As used herein with regard to DNA sequences or
35   segments, the phrase "operatively linked" means the

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sequences or segments have been covalently joined,
preferably by conventional phosphodiester bonds, into
one strand of DNA, whether in single or double
stranded form.

5   The choice of vector to which a cassette of this
invention is operatively linked depends directly, as
is well known in the art, on the functional properties
desired, e.g., vector replication and protein
expression, and the host cell to be transformed, these
10   being limitations inherent in the art of constructing
recombinant DNA molecules.

In preferred embodiments, the vector utilized
includes a prokaryotic replicon i.e., a DNA sequence
having the ability to direct autonomous replication
15   and maintenance of the recombinant DNA molecule extra
chromosomally in a prokaryotic host cell, such as a
bacterial host cell, transformed therewith. Such
replicons are well known in the art. In addition,
those embodiments that include a prokaryotic replicon
20   also include a gene whose expression confers a
selective advantage, such as drug resistance, to a
bacterial host transformed therewith. Typical
bacterial drug resistance genes are those that confer
resistance to ampicillin or tetracycline. Vectors
25   typically also contain convenient restriction sites
for insertion of translatable DNA sequences.
Exemplary vectors are the plasmids pUC8, pUC9, pBR322,
and pBR329 available from BioRad Laboratories,
(Richmond, CA) and pPL and pKK223 available from
30   Pharmacia, (Piscataway, NJ).

A sequence of nucleotides adapted for directional
ligation, i.e., a polylinker, is a region of the DNA
expression vector that (1) operatively links for
replication and transport the upstream and downstream
35   translatable DNA sequences and (2) provides a site or

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means for directional ligation of a DNA sequence into
the vector. Typically, a directional polylinker is a
sequence of nucleotides that defines two or more
restriction endonuclease recognition sequences, or

5   restriction sites. Upon restriction cleavage, the two
sites yield cohesive termini to which a translatable
DNA sequence can be ligated to the DNA expression
vector. Preferably, the two restriction sites
provide, upon restriction cleavage, cohesive termini

10   that are non-complementary and thereby permit
directional insertion of a translatable DNA sequence
into the cassette. In one embodiment, the directional
ligation means is provided by nucleotides present in
the upstream translatable DNA sequence, downstream

15   translatable DNA sequence, or both. In another
embodiment, the sequence of nucleotides adapted for
directional ligation comprises a sequence of
nucleotides that defines multiple directional cloning
means. Where the sequence of nucleotides adapted for

20   directional ligation defines numerous restriction
sites, it is referred to as a multiple cloning site.

        A translatable DNA sequence is a linear series of
nucleotides that provide an uninterrupted series of at
least 8 codons that encode a polypeptide in one

25   reading frame.

        An upstream translatable DNA sequence encodes a
prokaryotic secretion signal. The secretion signal is
a leader peptide domain of protein that targets the
protein to the periplasmic membrane of gram negative

30   bacteria.

        A preferred secretion signal is a pelB secretion
signal. The predicted amino acid residue sequences of
the secretion signal domain from two pelB gene product
variants from _Erwinia carotova_ are shown in Table 1 as

35   described by Lei, et al., _Nature_, 331:543-546 (1988).

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A particularly preferred pelB secretion signal is also shown in Table 1.

The leader sequence of the pelB protein has previously been used as a secretion signal for fusion proteins. Better et al., Science, 240:1041-1043 (1988); Sastry et al., Proc. Natl. Acad. Sci. USA, 86:5728-5732 (1989); and Mullinax et al., Proc. Natl. Acad. Sci. USA, 87:8095-8099 (1990).

Amino acid residue sequences for other secretion signal polypeptide domains from E. coli useful in this invention are also listed in Table 1. Oliver, In Neidhard, F.C. (ed.), Escherichia coli and Salmonella Typhimurium, American Society for Microbiology, Washington, D.C., 1:56-69 (1987).

A translatable DNA sequence encoding the pelB secretion signal having the amino acid residue sequence shown in SEQ. ID. NO. 5 is a preferred DNA sequence for inclusion in a DNA expression vector of this invention.

A downstream translatable DNA sequence encodes a filamentous phage membrane anchor. Preferred membrane anchors are obtainable from filamentous phage M13, f1, fd, and the like equivalent filamentous phage. Preferred membrane anchor domains are found in the coat proteins encoded by gene III and gene VIII. Thus, a downstream translatable DNA sequence encodes an amino acid residue sequence that corresponds, and preferably is identical, to the membrane anchor domain of either a filamentous phage gene III or gene VIII coat polypeptide.

The membrane anchor domain of a filamentous phage coat protein is a portion of the carboxy terminal region of the coat protein and includes a region of hydrophobic amino acid residues for spanning a lipid bilayer membrane, and a region of charged amino acid

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Table 1

Leader Sequences

	SEQ ID NO	Type	Amino Acid Residue Sequence
5	(5)	PelB[1]	MetLysTyrLeuLeuProThrAlaAlaAlaGlyLeuLeu LeuLeuAlaAlaGlnProAlaMet
	(6)	pelB[2]	MetLysTyrLeuLeuProThrAlaAlaAlaGlyLeuLeu LeuLeuAlaAlaGlnProAlaGlnProAlaMetAla
	(7)	pelB[3]	MetLysSerLeuIleThrProIleAlaAlaGlyLeuLeu LeuAlaPheSerGlnTyrSerLeuAla
10	(8)	MalE[4]	MetLysIleLysThrGlyAlaArgIleLeuAlaLeuSer AlaLeuThrThrMetMetPheSerAlaSerAlaLeuAla LysIle
	(9)	OmpF[4]	MetMetLysArgAsnIleLeuAlaValIleValProAla LeuLeuValAlaGlyThrAlaAsnAlaAlaGlu
15	(10)	PhoA[4]	MetLysGlnSerThrIleAlaLeuAlaLeuLeuProLeu LeuPheThrProValThrLysAlaArgThr
	(11)	Bla[4]	MetSerIleGlnHisPheArgValAlaLeuIleProPhe PheAlaAlaPheCysLeuProValPheAlaHisPro
20	(12)	LamB[4]	MetMetIleThrLeuArgLysLeuProLeuAlaValAla ValAlaAlaGlyValMetSerAlaGlnAlaMetAlaVal Asp
	(13)	Lpp[4]	MetLysAlaThrLysLeuValLeuGlyAlaValIleLeu GlySerThrLeuLeuAlaGlyCysSer
25	(14)	cpVIII[5]	MetLysLysSerLeuValLeuLysAlaSerValAlaVal AlaThrLeuValProMetLeuSerPheAla
	(15)	cpIII[6]	MetLysLysLeuLeuPheAlaIleProLeuValValPro PheTyrSerHisSer

30   [1] pelB used in this invention
      [2] pelB from _Erwinia carotovora_ gene
      [3] pelB from _Erwinia carotovora_ EC 16 gene
      [4] leader sequences from _E. coli_
      [5] leader sequence for cpVIII
35   [6] leader sequence for cpIII

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residues normally found at the cytoplasmic face of the
membrane and extending away from the membrane.

In the phage f1, gene VIII coat protein's
membrane spanning region comprises residue Trp-26
5   through Lys-40, and the cytoplasmic region comprises
the carboxy-terminal 11 residues from 41 to 52.
Ohkawa et al., J. Biol. Chem., 256:9951-9958 (1981).
An exemplary membrane anchor would consist of residues
26 to 40 of cpVIII.

10   Thus, the amino acid residue sequence of a
preferred membrane anchor domain is derived from the
M13 filamentous phage gene III coat protein (also
designated cpIII). A preferred cpIII-derived membrane
anchor has a sequence shown in SEQ ID NO. 16 from
15   residue 1 to residue 211. Gene III coat protein is
present on a mature filamentous phage at one end of
the phage particle with typically about 4 to 6 copies
of the coat protein.

The amino acid residue sequence of another
20   preferred membrane anchor domain is derived from the
M13 filamentous phage gene VIII coat protein (also
designated cpVIII). A preferred cpVIII-derived
membrane anchor has a sequence shown in SEQ ID NO. 17
from residue 1 to residue 50. Gene VIII coat protein
25   is present on a mature filamentous phage over the
majority of the phage particle with typically about
2500 to 3000 copies of the coat protein.

For detailed descriptions of the structure of
filamentous phage particles, their coat proteins and
30   particle assembly, see the reviews by Rached et al.,
Microbiol. Rev., 50:401-427 (1986); and Model et al.,
in "The Bacteriophages: Vol. 2", R. Calendar, ed.
Plenum Publishing Co., pp. 375-456, (1988).

A cassette in a DNA expression vector of this
35   invention is the region of the vector that forms, upon

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insertion of a translatable DNA sequence (insert DNA),
a sequence of nucleotides capable of expressing, in an
appropriate host, a fusion polypeptide of this
invention. The expression-competent sequence of
5   nucleotides is referred to as a cistron. Thus, the
cassette comprises DNA expression control elements
operatively linked to the upstream and downstream
translatable DNA sequences. A cistron is formed when
a translatable DNA sequence is directionally inserted
10   (directionally ligated) between the upstream and
downstream sequences via the sequence of nucleotides
adapted for that purpose. The resulting three
translatable DNA sequences, namely the upstream, the
inserted and the downstream sequences, are all
15   operatively linked in the same reading frame.

DNA expression control sequences comprise a set
of DNA expression signals for expressing a structural
gene product and include both 5' and 3' elements, as
is well known, operatively linked to the cistron such
20   that the cistron is able to express a structural gene
product. The 5' control sequences define a promoter
for initiating transcription and a ribosome binding
site operatively linked at the 5' terminus of the
upstream translatable DNA sequence.

25   To achieve high levels of gene expression in E.
coli, it is necessary to use not only strong promoters
to generate large quantities of mRNA, but also
ribosome binding sites to ensure that the mRNA is
efficiently translated. In E. coli, the ribosome
30   binding site includes an initiation codon (AUG) and a
sequence 3-9 nucleotides long located 3-11 nucleotides
upstream from the initiation codon [Shine et al.,
Nature, 254:34 (1975)]. The sequence, AGGAGGU, which
is called the Shine-Dalgarno (SD) sequence, is
35   complementary to the 3' end of E. coli 16S mRNA.

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Binding of the ribosome to mRNA and the sequence at
the 3' end of the mRNA can be affected by several
factors:

(i) The degree of complementarity between
the SD sequence and 3' end of the 16S tRNA.

(ii) The spacing and possibly the DNA
sequence lying between the SD sequence and the AUG
[Roberts et al., Proc. Natl. Acad. Sci. USA, 76:760
(1979a); Roberts et al., Proc. Natl. Acad. Sci. USA,
76:5596 (1979b); Guarente et al., Science, 209:1428
(1980); and Guarente et al., Cell, 20:543 (1980).]
Optimization is achieved by measuring the level of
expression of genes in plasmids in which this spacing
is systematically altered. Comparison of different
mRNAs shows that there are statistically preferred
sequences from positions -20 to +13 (where the A of
the AUG is position 0) [Gold et al., Annu. Rev.
Microbiol., 35:365 (1981)]. Leader sequences have
been shown to influence translation dramatically
(Roberts et al., 1979 a, b supra).

(iii) The nucleotide sequence following the
AUG, which affects ribosome binding [Taniguchi et al.,
J. Mol. Biol., 118:533 (1978)].

Useful ribosome binding sites are shown in Table
2 below.

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Table 2
Ribosome Binding Sites*

		SEQ. ID. NO.		
5	1.	(18)	5'	AAUCUU<u>GGAGG</u>CUUUUUU<u>AUG</u>GUUCGUUCU
	2.	(19)	5'	UAAC<u>UAAGGA</u>UGAAAUGC<u>AUG</u>UCUAAGACA
	3.	(20)	5'	UCCU<u>AGGAGG</u>UUUGACCU<u>AUG</u>CGAGCUUUU
	4.	(21)	5'	AUGUAC<u>UAAGGAGG</u>UUGU<u>AUG</u>GAACAACGC

* Sequences of initiation regions for protein
    synthesis in four phage mRNA molecules are
    underlined.

AUG = initiation codon (double underlined)

1. = Phage φX174 gene-A protein

2. = Phage Qβ replicase

3. = Phage R17 gene-A protein

4. = Phage lambda gene-cro protein

The 3' control sequences define at least one termination (stop) codon in frame with and operatively linked to the downstream translatable DNA sequence.

Thus, a DNA expression vector of this invention provides a system for cloning translatable DNA sequences into the cassette portion of the vector to produce a cistron capable of expressing a fusion polypeptide of this invention.

In preferred embodiments, a DNA expression vector provides a system for independently cloning (inserting) two translatable DNA sequences into two separate cassettes present in the vector, to form two separate cistrons for expressing both polypeptides of a heterodimeric receptor, or the ligand binding portions of the polypeptides that comprise a heterodimeric receptor. The DNA expression vector for expressing two cistrons is referred to as a

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dicistronic expression vector.

Thus, a preferred DNA expression vector of this invention comprises, in addition to the cassette previously described in detail, a second cassette for expressing a second fusion polypeptide. The second cassette includes a second translatable DNA sequence that encodes a secretion signal, as defined herein before, operatively linked at its 3' terminus via a sequence of nucleotides adapted for directional ligation to a downstream DNA sequence of the vector that typically defines at least one stop codon in the reading frame of the cassette. The second translatable DNA sequence is operatively linked at its 5' terminus to DNA expression control sequences forming the 5' elements defined above. The second cassette is capable, upon insertion of a translatable DNA sequence (insert DNA), of expressing the second fusion polypeptide comprising a fusion of the secretion signal with a polypeptide coded by the insert DNA.

In a preferred embodiment, a DNA expression vector is designed for convenient manipulation in the form of a filamentous phage particle encapsulating a genome according to the teachings of the present invention. In this embodiment, a DNA expression vector further contains a nucleotide sequence that defines a filamentous phage origin of replication such that the vector, upon presentation of the appropriate genetic complementation, can replicate as a filamentous phage in single stranded replicative form and be packaged into filamentous phage particles. This feature provides the ability of the DNA expression vector to be packaged into phage particles for subsequent segregation of the particle, and vector contained therein, away from other particles that

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comprise a population of phage particles.

A filamentous phage origin of replication is a
region of the phage genome, as is well known, that
defines sites for initiation of replication,
5   termination of replication and packaging of the
replicative form produced by replication. See, for
example, Rasched et al., _Microbiol. Rev._, 50:401-427
(1986); and Horiuchi, _J. Mol. Biol._, 188:215-223
(1986).
10   A preferred filamentous phage origin of
replication for use in the present invention is a M13,
f1 or fd phage origin of replication. Particularly
preferred is a filamentous phage origin of replication
having a sequence shown in SEQ ID NO. 117 and
15   described by Short et al., _Nucl. Acids Res._, 16:7583-
7600 (1988). Preferred DNA expression vectors are the
dicistronic expression vectors pCOMB8, pCKAB8, pCOMB2-
8, pCOMB3, pCKAB3, pCOMB2-3 and pCOMB2-3' described in
Example 1.
20   Insofar as a vector of this invention may be
manipulated to contain an insert DNA, thereby having
the capacity to express a fusion polypeptide, one
embodiment contemplates the previously described
vectors containing an insert DNA. Particularly
25   preferred vectors containing antibody genes are
described in the Examples.

        D.   Polypeptides
             In another embodiment, the present invention
30   contemplates a polypeptide comprising an insert domain
flanked by an amino-terminal secretion signal domain
and a carboxy-terminal filamentous phage coat protein
membrane anchor domain.
        The polypeptide is a fusion polypeptide having a
35   receptor domain comprised of an amino acid residue

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sequence that defines the ligand (epitope) binding
domain of a receptor protein positioned between a
prokaryotic secretion signal domain and a filamentous
phage membrane anchor domain. That is, the insert
domain in the fusion polypeptide is the ligand-binding
domain of a receptor and is also referred to as a
ligand-binding receptor polypeptide.

Insofar as the polypeptide has a receptor domain,
it is also referred to herein as a receptor. In other
preferred embodiments the secretion signal domain is a
pelB secretion signal as described herein. In
addition, it is preferred that the membrane anchor
domain be derived from the filamentous phage cpIII or
cpVIII proteins as described herein.

In preferred embodiments, the receptor protein is
a polypeptide chain of a ligand-binding heterodimeric
receptor. More preferably the heterodimeric receptor
is an epitope-binding complex. ·

Preferred heterodimeric receptors include
immunoglobulins, major histocompatibility antigens of
class I or II, lymphocyte receptors, integrins and the
like heterodimeric receptors. Immunoglobulins
(antibody molecules) can be in the form of Fab or Fv
fragments, or other portions of an antibody molecule
that contain regions of the variable domain of the
heavy and light chains.

In one embodiment, a polypeptide of this
invention has an amino acid residue sequence that can
be represented by the formula, shown in the direction
of amino- to carboxy terminus:

(F1) $NH_2-O-(U)_m-V-(X)_n-Z-COOH$,
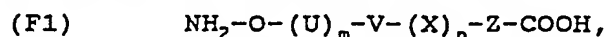
where O represents an amino acid residue sequence
defining a secretion signal, U represents a first
spacer polypeptide, V represents an amino acid residue
sequence defining a receptor domain (a ligand-binding

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receptor polypeptide), X represents a second spacer
polypeptide, and Z represents an amino acid residue
sequence defining a filamentous phage coat protein
membrane anchor, with the proviso that m is the
integer 0 or 1 such that when m is 0, U is not
present, and when m is 1, U is present, and n is 0 or
1 such that when n is 0, X is not present and when n
is 1, X is present.

          In the formula (F1), the secretion signal and the
filamentous phage coat protein membrane anchor are as
defined herein above. Thus, a preferred polypeptide
comprises an antibody variable chain domain-derived
polypeptide operatively linked at its amino-terminus
to the secretion signal and operatively linked at its
carboxy-terminus to the membrane anchor.

          A preferred polypeptide of this embodiment
consists essentially of an antibody heavy chain
polypeptide as the variable domain. In this regard
"consists essentially of" means that the polypeptide
does not contain an antibody light chain polypeptide,
or portion thereof. Particularly preferred is a
polypeptide according to formula (F1) where Z defines
the cpIII or cpVIII membrane anchor as described
herein. In another preferred embodiment the secretion
signal is the pelB secretion signal.

          As used herein with regard to polypeptides, the
phrase "operatively linked" means that polypeptide
fragments, or protein domains represented by
polypeptides, have been covalently joined into a
single polypeptide polymer, preferably by conventional
amide bonds between the adjacent amino acids being
linked in the polypeptide.

          In one embodiment, V is an amino acid residue
sequence that defines the ligand binding domain of a
chain of a heterodimeric receptor molecule, and

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preferably is an immunoglobulin variable region
polypeptide. In a particularly preferred polypeptide
V is a V_H or V_L polypeptide. Most preferred is a
polypeptide where V is an immunoglobulin V_H
5   polypeptide (antibody heavy chain polypeptide), and m
and n are both zero.

In another embodiment, U or X can define a
proteolytic cleavage site, such as the sequence of
amino acids found in a precursor protein, such as
10   prothrombin, factor X and the like, that defines the
site of cleavage of the polypeptide. A fusion
polypeptide having a cleavage site provides a means to
purify the polypeptide away from the phage particle to
which it is attached.

15   The polypeptide spacers U and X can each have any
sequence of amino acid residues of from about 1 to 6
amino acid residues in length. Typically the spacer
residues are present in a polypeptide to accommodate
the continuous reading frame that is required when a
20   polypeptide is produced by the methods disclosed
herein using a DNA expression vector of this
invention.

A receptor of the present invention assumes a
conformation having a binding site specific for, as
25   evidenced by its ability to be competitively
inhibited, a preselected or predetermined ligand such
as an antigen, hapten, enzymatic substrate and the
like. In one embodiment, a receptor of this invention
is a ligand binding polypeptide that forms an antigen
30   binding site which specifically binds to a preselected
antigen to form a complex having a sufficiently strong
binding between the antigen and the binding site for
the complex to be isolated. When the receptor is an
antigen binding polypeptide its affinity or avidity is
35   generally greater than 10^5 M^{-1} more usually greater

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than 10^6 and preferably greater than 10^8 M^{-1}.

In another embodiment, a receptor of the subject invention binds a substrate and catalyzes the formation of a product from the substrate. While the topology of the ligand binding site of a catalytic receptor is probably more important for its preselected activity than its affinity (association constant or pKa) for the substrate, the subject catalytic receptors have an association constant for the preselected substrate generally greater than 10^3 M^{-1}, more usually greater than 10^5 M^{-1} or 10^6 M^{-1} and preferably greater than 10^7 M^{-1}.

Preferably the receptor produced by the subject invention is heterodimeric and is therefore normally comprised of two different polypeptide chains, which together assume a conformation having a binding affinity, or association constant for the preselected ligand that is different, preferably higher, than the affinity or association constant of either of the polypeptides alone, i.e., as monomers. The heterodimeric receptor is referred to as a ligand-binding heterodimeric receptor to connote its ability to bind ligand.

Thus, a preferred embodiment contemplates a ligand-binding heterodimeric receptor comprising first and second polypeptides. The first polypeptide is flanked by an amino-terminal prokaryotic secretion signal domain and a carboxy-terminal filamentous phage membrane anchor domain. The second polypeptide is fused to an amino-terminal prokaryotic secretion signal domain. A particularly preferred ligand-binding heterodimeric receptor utilizes a prokaryotic secretion signal as described herein. Additionally, a preferred ligand-binding heterodimeric receptor contains a membrane anchor-derived from cpIII or

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cpVIII as described herein.

A ligand-binding heterodimeric receptor is referred to as an epitope-binding complex to connote that the complex has a capacity to bind an epitope present on a ligand, and to connote that the heterodimeric receptor is formed by the association (complexation) of two polypeptides as described herein.

One or both of the different polypeptide chains is preferably derived from the variable region of the light and heavy chains of an immunoglobulin. Typically, polypeptides comprising the light (V_L) and heavy (V_H) variable regions are employed together for binding the preselected ligand.

Thus, one embodiment contemplates a ligand-binding heterodimeric receptor in which the first polypeptide is an antibody heavy chain polypeptide and the second polypeptide is a light chain polypeptide. An alternative embodiment contemplates a ligand-binding heterodimeric receptor in which the first polypeptide is an antibody light chain polypeptide and the second is an antibody heavy chain polypeptide.

A receptor produced by the subject invention can be active in monomeric as well as multimeric forms, either homomeric or heteromeric, preferably heterodimeric. For example, V_H and V_L ligand binding polypeptide produced by the present invention can be advantageously combined in the heterodimer to modulate the activity of either or to produce an activity unique to the heterodimer.

Where the individual ligand polypeptides are referred to as V_H and V_L, the heterodimer can be referred to as a Fv. However, it should be understood that a V_H may contain in addition to the V_H, substantially all or a portion of the heavy chain

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constant region. Similarly, a V_L may contain, in
addition to the V_L, substantially all or a portion of
the light chain constant region. A heterodimer
comprised of a V_H containing a portion of the heavy
chain constant region and a V_L containing
substantially all of the light chain constant region
is termed a Fab fragment. The production of Fab can
be advantageous in some situations because the
additional constant region sequences contained in a
Fab as compared to a Fv can stabilize the V_H and V_L
interaction. Such stabilization can cause the Fab to
have higher affinity for antigen. In addition the Fab
is more commonly used in the art and thus there are
more commercial antibodies available to specifically
recognize a Fab in screening procedures.

The individual V_H and V_L polypeptides can be
produced in lengths equal to or substantially equal to
their naturally occurring lengths. However, in
preferred embodiments, the V_H and V_L polypeptides will
generally have fewer than 125 amino acid residues,
more usually fewer than about 120 amino acid residues,
while normally having greater than 60 amino acid
residues, usually greater than about 95 amino acid
residues, more usually greater than about 100 amino
acid residues. Preferably, the V_H will be from about
110 to about 230 amino acid residues in length while
V_L will be from about 95 to about 214 amino acid
residues in length. V_H and V_L chains sufficiently
long to form Fabs are preferred.

The amino acid residue sequences will vary
widely, depending upon the particular idiotype
involved. Usually, there will be at least two
cysteines separated by from about 60 to 75 amino acid
residues and joined by a disulfide bond. The
polypeptides produced by the subject invention will

normally be substantial copies of idiotypes of the
variable regions of the heavy and/or light chains of
immunoglobulins, but in some situations a polypeptide
may contain random mutations in amino acid residue

5   sequences in order to advantageously improve the
desired activity.

In some situations, it is desirable to provide
for covalent cross linking of the V_H and V_L
polypeptides, which can be accomplished by providing

10   cysteine resides at the carboxyl termini. The
polypeptide will normally be prepared free of the
immunoglobulin constant regions, however a small
portion of the J region may be included as a result of
the advantageous selection of DNA synthesis primers.

15   The D region will normally be included in the
transcript of the V_H.

Typically the C terminus region of the V_H and V_L
polypeptides will have a greater variety of sequences
than the N terminus and, based on the present

20   strategy, can be further modified to permit a
variation of the normally occurring V_H and V_L chains.
A synthetic polynucleotide can be employed to vary one
or more amino acid in a hypervariable region.

25   E.   Methods for Producing a Library
                1.   General Rationale
                      In one embodiment the present invention
provides a system for the simultaneous cloning and
screening of preselected ligand-binding specificities

30   from gene repertoires using a single vector system.
This system provides linkage of cloning and screening
methodologies and has two requirements. First, that
expression of the polypeptide chains of a
heterodimeric receptor in an _in vitro_ expression host

35   such as _E. coli_ requires coexpression of the two

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polypeptide chains in order that a functional
heterodimeric receptor can assemble to produce a
receptor that binds ligand. Second, that screening of
isolated members of the library for a preselected
5   ligand-binding capacity requires a means to correlate
(a linkage) the binding capacity of an expressed
receptor molecule with a convenient means to isolate
the gene that encodes the member from the library.
        Linkage of expression and screening is
10   accomplished by the combination of targeting of a
fusion polypeptide into the periplasm of a bacterial
cell to allow assembly of a functional receptor, and
the targeting of a fusion polypeptide onto the coat of
a filamentous phage particle during phage assembly to
15   allow for convenient screening of the library member
of interest. Periplasmic targeting is provided by the
presence of a secretion signal domain in a fusion
polypeptide of this invention. Targeting to a phage
particle is provided by the presence of a filamentous
20   phage coat protein membrane anchor domain (i.e., a
cpIII- or cpVIII-derived membrane anchor domain) in a
fusion polypeptide of this invention.
        The present invention describes in one embodiment
a method for producing a library of DNA molecules,
25   each DNA molecule comprising a cistron for expressing
a fusion polypeptide on the surface of a filamentous
phage particle. The method comprises the steps of (a)
forming a ligation admixture by combining in a
ligation buffer (i) a repertoire of polypeptide
30   encoding genes and (ii) a plurality of DNA expression
vectors in linear form adapted to form a fusion
polypeptide expressing cistron, and (b) subjecting the
admixture to ligation conditions for a time period
sufficient for the repertoire of genes to become
35   operatively linked (ligated) to the plurality of

vectors to form the library.

In this embodiment, the repertoire of polypeptide encoding genes are in the form of double-stranded (ds) DNA and each member of the repertoire has cohesive termini adapted for directional ligation. In addition, the plurality of DNA expression vectors are each linear DNA molecules having upstream and downstream cohesive termini that are (a) adapted for directionally receiving the polypeptide genes in a common reading frame, and (b) operatively linked to respective upstream and downstream translatable DNA sequences. The upstream translatable DNA sequence encodes a secretion signal, preferably a pelB secretion signal, and the downstream translatable DNA sequence encodes a filamentous phage coat protein membrane anchor as described herein for a polypeptide of this invention. The translatable DNA sequences are also operatively linked to respective upstream and downstream DNA expression control sequences as defined for a DNA expression vector described herein.

The library so produced can be utilized for expression and screening of the fusion polypeptides encoded by the resulting library of cistrons represented in the library by the expression and screening methods described herein.

2.   Production of Gene Repertoires

A gene repertoire is a collection of different genes, preferably polypeptide-encoding genes (polypeptide genes), and may be isolated from natural sources or can be generated artificially. Preferred gene repertoires are comprised of conserved genes. Particularly preferred gene repertoires comprise either or both genes that code for the members of a dimeric receptor molecule.

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A gene repertoire useful in practicing the
present invention contains at least 10^3, preferably at
least 10^4, more preferably at least 10^5, and most
preferably at least 10^7 different genes. Methods for
5   evaluating the diversity of a repertoire of genes is
well known to one skilled in the art.

Thus, in one embodiment, the present invention
contemplates a method of isolating a pair of genes
coding for a dimeric receptor having a preselected
10   activity from a repertoire of conserved genes.
Additionally, expressing the cloned pair of genes and
isolating the resulting expressed dimeric receptor
protein is also described. Preferably, the receptor
will be a heterodimeric polypeptide capable of binding
15   a ligand, such as an antibody molecule or
immunologically active portion thereof, a cellular
receptor, or a cellular adhesion protein coded for by
one of the members of a family of conserved genes,
i.e., genes containing a conserved nucleotide sequence
20   of at least about 10 nucleotides in length.

Exemplary conserved gene families encoding
different polypeptide chains of a dimeric receptor are
those coding for immunoglobulins, major
histocompatibility complex antigens of class I or II,
25   lymphocyte receptors, integrins and the like.

A gene can be identified as belonging to a
repertoire of conserved genes using several methods.
For example, an isolated gene may be used as a
hybridization probe under low stringency conditions to
30   detect other members of the repertoire of conserved
genes present in genomic DNA using the methods
described by Southern, _J. Mol. Biol._, 98:503 (1975).
If the gene used as a hybridization probe hybridizes
to multiple restriction endonuclease fragments of the
35   genome, that gene is a member of a repertoire of

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conserved genes.

Immunoglobulins

The immunoglobulins, or antibody molecules, are a
large family of molecules that include several types
of molecules, such as IgD, IgG, IgA, IgM and IgE. The
antibody molecule is typically comprised of two heavy
(H) and light (L) chains with both a variable (V) and
constant (C) region present on each chain as shown in
Figure 1. Schematic diagrams of human IgG heavy chain
and human kappa light chain are shown in Figures 2A
and 2B, respectively. Several different regions of an
immunoglobulin contain conserved sequences useful for
isolating an immunoglobulin repertoire. Extensive
amino acid and nucleic acid sequence data displaying
exemplary conserved sequences is compiled for
immunoglobulin molecules by Kabat et al., in Sequences
of Proteins of Immunological Interest, National
Institutes of Health, Bethesda, MD, 1987.

The C region of the H chain defines the
particular immunoglobulin type. Therefore the
selection of conserved sequences as defined herein
from the C region of the H chain results in the
preparation of a repertoire of immunoglobulin genes
having members of the immunoglobulin type of the
selected C region.

The V region of the H or L chain typically
comprises four framework (FR) regions each containing
relatively lower degrees of variability that includes
lengths of conserved sequences. The use of conserved
sequences from the FR1 and FR4 (J region) framework
regions of the V_H chain is a preferred exemplary
embodiment and is described herein in the Examples.
Framework regions are typically conserved across
several or all immunoglobulin types and thus conserved

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sequences contained therein are particularly suited
for preparing repertoires having several
immunoglobulin types.

Major Histocompatibility Complex

The major histocompatibility complex (MHC) is a
large genetic locus that encodes an extensive family
of proteins that include several classes of molecules
referred to as class I, class II or class III MHC
molecules. Paul et al., in Fundamental Immunology,
Raven Press, NY, pp. 303-378 (1984).

Class I MHC molecules are a polymorphic group of
transplantation antigens representing a conserved
family in which the antigen is comprised of a heavy
chain and a non-MHC encoded light chain. The heavy
chain includes several regions, termed the N, C1, C2,
membrane and cytoplasmic regions. Conserved sequences
useful in the present invention are found primarily in
the N, C1 and C2 regions and are identified as
continuous sequences of "invariant residues" in Kabat
et al., supra.

Class II MHC molecules comprise a conserved
family of polymorphic antigens that participate in
immune responsiveness and are comprised of an alpha
and a beta chain. The genes coding for the alpha and
beta chain each include several regions that contain
conserved sequences suitable for producing MHC class
II alpha or beta chain repertoires. Exemplary
conserved nucleotide sequences include those coding
for amino acid residues 26-30 of the A1 region,
residues 161-170 of the A2 region and residues 195-206
of the membrane region, all of the alpha chain.
Conserved sequences are also present in the B1, B2 and
membrane regions of the beta chain at nucleotide
sequences coding for amino acid residues 41-45, 150-

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162 and 200-209, respectively.

<u>Lymphocyte Receptors and Cell Surface Antigens</u>

Lymphocytes contain several families of proteins
on their cell surfaces including the T-cell receptor,
Thy-1 antigen and numerous T-cell surface antigens
including the antigens defined by the monoclonal
antibodies OKT4 (leu3), OKT5/8 (leu2), OKT3, OKT1
(leu1), OKT 11 (leu5) OKT6 and OKT9. Paul, <u>supra</u> at
pp. 458-479.

The T-cell receptor is a term used for a family
of antigen binding molecules found on the surface of
T-cells. The T-cell receptor as a family exhibits
polymorphic binding specificity similar to
immunoglobulins in its diversity. The mature T-cell
receptor is comprised of alpha and beta chains each
having a variable (V) and constant (C) region. The
similarities that the T-cell receptor has to
immunoglobulins in genetic organization and function
shows that T-cell receptor contains regions of
conserved sequence. Lai et al., <u>Nature</u>, 331:543-546
(1988).

Exemplary conserved sequences include those
coding for amino acid residues 84-90 of alpha chain,
amino acid residues 107-115 of beta chain, and amino
acid residues 91-95 and 111-116 of the gamma chain.
Kabat et al., <u>supra</u>, p. 279.

<u>Integrins And Adhesions</u>

Adhesive proteins involved in cell attachment are
members of a large family of related proteins termed
integrins. Integrins are heterodimers comprised of a
beta and an alpha subunit. Members of the integrin
family include the cell surface glycoproteins platelet
receptor GpIIb-IIIa, vitronectin receptor (VnR),

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fibronectin receptor (FnR) and the leukocyte adhesion
receptors LFA-1, Mac-1, Mo-1 and 60.3. Rouslahti et
al., Science, 238:491-497 (1987). Nucleic acid and
protein sequence data demonstrates regions of
5   conserved sequences exist in the members of these
families, particularly between the beta chain of
GpIIb-IIIa, VnR and FnR, and between the alpha subunit
of VnR, Mac-1, LFA-1, FnR and GpIIb-IIIa. Suzuki et
al., Proc. Natl. Acad. Sci. USA, 83:8614-8618, 1986;
10   Ginsberg et al., J. Biol. Chem., 262:5437-5440, 1987.

Various well known methods can be employed to
produce a useful gene repertoire. For instance, V_H
and V_L gene repertoires can be produced by isolating
15   V_H- and V_L-coding mRNA from a heterogeneous population
of antibody producing cells, i.e., B lymphocytes (B
cells), preferably rearranged B cells such as those
found in the circulation or spleen of a vertebrate.
Rearranged B cells are those in which immunoglobulin
20   gene translocation, i.e., rearrangement, has occurred
as evidenced by the presence in the cell of mRNA with
the immunoglobulin gene V, D and J region transcripts
adjacently located thereon. Typically, the B cells
are collected in a 1-100 ml sample of blood which
25   usually contains 10^6 B cells/ml.

In some cases, it is desirable to bias a
repertoire for a preselected activity, such as by
using as a source of nucleic acid cells (source cells)
from vertebrates in any one of various stages of age,
30   health and immune response. For example, repeated
immunization of a healthy animal prior to collecting
rearranged B cells results in obtaining a repertoire
enriched for genetic material producing a receptor of
high affinity. Mullinax et al., Proc. Natl. Acad.
35   Sci. USA, 87:8095-8099 (1990). Conversely, collecting

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rearranged B cells from a healthy animal whose immune
system has not been recently challenged (i.e., a naive
immune system) results in producing a repertoire that
is not biased towards the production of high affinity
5   V_H and/or V_L polypeptides.

It should be noted the greater the genetic
heterogeneity of the population of cells for which the
nucleic acids are obtained, the greater the diversity
of the immunological repertoire (comprising V_H- and
10   V_L-coding genes) that will be made available for
screening according to the method of the present
invention. Thus, cells from different individuals,
particularly those having an immunologically
significant age difference, and cells from individuals
15   of different strains, races or species can be
advantageously combined to increase the heterogeneity
(diversity) of a repertoire.

Thus, in one preferred embodiment, the source
cells are obtained from a vertebrate, preferably a
20   mammal, which has been immunized or partially
immunized with an antigenic ligand (antigen) against
which activity is sought, i.e., a preselected antigen.
The immunization can be carried out conventionally.
Antibody titer in the animal can be monitored to
25   determine the stage of immunization desired, which
stage corresponds to the amount of enrichment or
biasing of the repertoire desired. Partially
immunized animals typically receive only one
immunization and cells are collected from those
30   animals shortly after a response is detected. Fully
immunized animals display a peak titer, which is
achieved with one or more repeated injections of the
antigen into the host mammal, normally at 2 to 3 week
intervals. Usually three to five days after the last
35   challenge, the spleen is removed and the genetic

repertoire of the splenocytes, about 90% of which are rearranged B cells, is isolated using standard procedures. See, <u>Current Protocols in Molecular Biology</u>, Ausubel et al., eds., John Wiley & Sons, NY.

5 Nucleic acids coding for V_H and V_L polypeptides can be derived from cells producing IgA, IgD, IgE, IgG or IgM, most preferably from IgM and IgG, producing cells.

Methods for preparing fragments of genomic DNA

10 from which immunoglobulin variable region genes can be cloned as a diverse population are well known in the art. See for example Herrmann et al., <u>Methods In Enzymol.</u>, 152:180-183, (1987); Frischauf, <u>Methods In Enzymol.</u>, 152:183-190 (1987); Frischauf, <u>Methods In</u>

15 <u>Enzymol.</u>, 152:190-199 (1987); and DiLella et al., <u>Methods In Enzymol.</u>, 152:199-212 (1987). (The teachings of the references cited herein are hereby incorporated by reference.)

The desired gene repertoire can be isolated from

20 either genomic material containing the gene expressing the variable region or the messenger RNA (mRNA) which represents a transcript of the variable region. The difficulty in using the genomic DNA from other than non-rearranged B lymphocytes is in juxtaposing the

25 sequences coding for the variable region, where the sequences are separated by introns. The DNA fragment(s) containing the proper exons must be isolated, the introns excised, and the exons then spliced in the proper order and in the proper

30 orientation. For the most part, this will be difficult, so that the alternative technique employing rearranged B cells will be the method of choice because the V, D and J immunoglobulin gene regions have translocated to become adjacent, so that the

35 sequence is continuous (free of introns) for the

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entire variable regions.

Where mRNA is utilized the cells will be lysed under RNase inhibiting conditions. In one embodiment, the first step is to isolate the total cellular mRNA. Poly A+ mRNA can then be selected by hybridization to an oligo-dT cellulose column. The presence of mRNAs coding for the heavy and/or light chain polypeptides can then be assayed by hybridization with DNA single strands of the appropriate genes. Conveniently, the sequences coding for the constant portion of the V_H and V_L can be used as polynucleotide probes, which sequences can be obtained from available sources. See for example, Early and Hood, Genetic Engineering, Setlow and Hollaender, eds., Vol. 3, Plenum Publishing Corporation, NY, (1981), pages 157-188; and Kabat et al., Sequences of Immunological Interest, National Institutes of Health, Bethesda, MD, (1987).

In preferred embodiments, the preparation containing the total cellular mRNA is first enriched for the presence of V_H and/or V_L coding mRNA. Enrichment is typically accomplished by subjecting the total mRNA preparation or partially purified mRNA product thereof to a primer extension reaction employing a polynucleotide synthesis primer as described herein. Exemplary methods for producing V_H and V_L gene repertoires using polynucleotide synthesis primers are described in PCT Application No. PCT/US 90/02836 (International Publication No. WO 90/14430). Particularly preferred methods for producing a gene repertoire rely on the use of preselected oligonucleotides as primers in a polymerase chain reaction (PCR) to form PCR reaction products as described herein.

In preferred embodiments, isolated B cells are immunized in vitro against a preselected antigen. In

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vitro immunization is defined as the clonal expansion
of epitope-specific B cells in culture, in response to
antigen stimulation. The end result is to increase
the frequency of antigen-specific B cells in the
immunoglobulin repertoire, and thereby decrease the
number of clones in an expression library that must be
screened to identify a clone expressing an antibody of
the desired specificity. The advantage of _in vitro_
immunization is that human monoclonal antibodies can
be generated against a limitless number of
therapeutically valuable antigens, including toxic or
weak immunogens. For example, antibodies specific for
the polymorphic determinants of tumor-associated
antigens, rheumatoid factors, and histocompatibility
antigens can be produced, which can not be elicited in
immunized animals. In addition, it may be possible to
generate immune responses which are normally
suppressed _in vivo_.

In vitro immunization can be used to give rise to
either a primary or secondary immune response. A
primary immune response, resulting from first time
exposure of a B cell to an antigen, results in clonal
expansion of epitope-specific cells and the secretion
of IgM antibodies with low to moderate apparent
affinity constants (10^6-10^8 M^{-1}). Primary immunization
of human splenic and tonsillar lymphocytes in culture
can be used to produce monoclonal antibodies against a
variety of antigens, including cells, peptides,
macromolecule, haptens, and tumor-associated antigens.
Memory B cells from immunized donors can also be
stimulated in culture to give rise to a secondary
immune response characterized by clonal expansion and
the production of high affinity antibodies ($>10^9$ M^{-1})
of the IgG isotype, particularly against viral
antigens by clonally expanding sensitized lymphocytes

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derived from seropositive individuals.

In one embodiment, peripheral blood lymphocytes are depleted of various cytolytic cells that appear to down-modulate antigen-specific B cell activation. When lysosome-rich subpopulations (natural killer cells, cytotoxic and suppressor T cells, monocytes) are first removed by treatment with the lysosmotropic methyl ester of leucine, the remaining cells (including B cells, T helper cells, accessory cells) respond antigen-specifically during _in vitro_ immunization. The lymphokine requirements for inducing antibody production in culture are satisfied by a culture supernatant from activated, irradiated T cells.

In addition to _in vitro_ immunization, cell panning (immunoaffinity absorption) can be used to further increase the frequency of antigen-specific B cells. Techniques for selecting B cell subpopulations via solid-phase antigen binding are well established. Panning conditions can be optimized to selectively enrich for B cells which bind with high affinity to a variety of antigens, including cell surface proteins. Panning can be used alone, or in combination with _in vitro_ immunization to increase the frequency of antigen-specific cells above the levels which can be obtained with either technique alone. Immunoglobulin expression libraries constructed from enriched populations of B cells are biased in favor of antigen-specific antibody clones, and thus, enabling identification of clones with the desired specificities from smaller, less complex libraries.

In one embodiment, donor peripheral blood lymphocytes (PBL) can be transferred into severe combined immunodeficiency (SCID) mice and then boosted _in vivo_ in the SCID mice to increase the immune

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response prior to harvesting the heavy and light chain
coding nucleic acids from the SCID mouse's B cells.
See for example, Duchosal et al, Nature, 355:258-262
(1992). In that report, human PBLs from a donor who
5   had anti-tetanus toxoid (TT) titers were boosted while
in the SCID mouse host. The resulting library of
370,000 clones yielded 2 phage particles expressing a
surface Fab able to bind TT.

10   3.   Preparation of Polynucleotide Primers
                 The term "polynucleotide" as used
herein in reference to primers, probes and nucleic
acid fragments or segments to be synthesized by primer
extension is defined as a molecule comprised of two or
15   more deoxyribonucleotide or ribonucleotides,
preferably more than 3. Its exact size will depend on
many factors, which in turn depends on the ultimate
conditions of use.

            The term "primer" as used herein refers to a
20   polynucleotide whether purified from a nucleic acid
restriction digest or produced synthetically, which is
capable of acting as a point of initiation of nucleic
acid synthesis when placed under conditions in which
synthesis of a primer extension product which is
25   complementary to a nucleic acid strand is induced,
i.e., in the presence of nucleotides and an agent for
polymerization such as DNA polymerase, reverse
transcriptase and the like, and at a suitable
temperature and pH. The primer is preferably single
30   stranded for maximum efficiency, but may alternatively
be in double stranded form. If double stranded, the
primer is first treated to separate it from its
complementary strand before being used to prepare
extension products. Preferably, the primer is a
35   polydeoxyribonucleotide. The primer must be

sufficiently long to prime the synthesis of extension
products in the presence of the agents for
polymerization. The exact lengths of the primers will
depend on may factors, including temperature and the

5   source of primer. For example, depending on the
complexity of the target sequence, a polynucleotide
primer typically contains 15 to 25 or more
nucleotides, although it can contain fewer
nucleotides. Short primer molecules generally require

10   cooler temperatures to form sufficiently stable hybrid
complexes with template.

The primers used herein are selected to be
"substantially" complementary to the different strands
of each specific sequence to be synthesized or

15   amplified. This means that the primer must be
sufficiently complementary to non-randomly hybridize
with its respective template strand. Therefore, the
primer sequence may or may not reflect the exact
sequence of the template. For example, a non-

20   complementary nucleotide fragment can be attached to
the 5' end of the primer, with the remainder of the
primer sequence being substantially complementary to
the strand. Such non-complementary fragments
typically code for an endonuclease restriction site.

25   Alternatively, non-complementary bases or longer
sequences can be interspersed into the primer,
provided the primer sequence has sufficient
complementarily with the sequence of the strand to be
synthesized or amplified to non-randomly hybridize

30   therewith and thereby form an extension product under
polynucleotide synthesizing conditions.

Primers of the present invention may also contain
a DNA-dependent RNA polymerase promoter sequence or
its complement. See for example, Krieg et al., Nucl.

35   Acids Res., 12:7057-70 (1984); Studier et al., J. Mol.

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Biol., 189:113-130 (1986); and _Molecular Cloning: A Laboratory Manual, Second Edition_, Maniatis et al., eds., Cold Spring Harbor, NY (1989).

When a primer containing a DNA-dependent RNA polymerase promoter is used the primer is hybridized to the polynucleotide strand to be amplified and the second polynucleotide strand of the DNA-dependent RNA polymerase promoter is completed using an inducing agent such as _E. coli_ DNA polymerase I, or the Klenow fragment of _E. coli_ DNA polymerase. The starting polynucleotide is amplified by alternating between the production of an RNA polynucleotide and DNA polynucleotide.

Primers may also contain a template sequence or replication initiation site for a RNA-directed RNA polymerase. Typical RNA-directed RNA polymerase include the QB replicase described by Lizardi et al., _Biotechnology_, 6:1197-1202 (1988). RNA-directed polymerases produce large numbers of RNA strands from a small number of template RNA strands that contain a template sequence or replication initiation site. These polymerases typically give a one million-fold amplification of the template strand as has been described by Kramer et al., _J. Mol. Biol._, 89:719-736 (1974).

The polynucleotide primers can be prepared using any suitable method, such as, for example, the phosphotriester or phosphodiester methods see Narang et al., _Meth. Enzymol._, 68:90, (1979); U.S. Patent No. 4,356,270; and Brown et al., _Meth. Enzymol._, 68:109, (1979).

The choice of a primer's nucleotide sequence depends on factors such as the distance on the nucleic acid from the region coding for the desired receptor, its hybridization site on the nucleic acid relative to

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any second primer to be used, the number of genes in
the repertoire it is to hybridize to, and the like.

        a.   <u>Primers for Producing</u>
            <u>Immunoglobulin Gene Repertoires</u>

V_H and V_L gene repertoires can be
separately prepared prior to their utilization in the
present invention. Repertoire preparation is
typically accomplished by primer extension, preferably
by primer extension in a polymerase chain reaction
(PCR) format.

To produce a repertoire of V_H-coding DNA homologs
by primer extension, the nucleotide sequence of a
primer is selected to hybridize with a plurality of
immunoglobulin heavy chain genes at a site
substantially adjacent to the V_H-coding region so that
a nucleotide sequence coding for a functional (capable
of binding) polypeptide is obtained. To hybridize to
a plurality of different V_H-coding nucleic acid
strands, the primer must be a substantial complement
of a nucleotide sequence conserved among the different
strands. Such sites include nucleotide sequences in
the constant region, any of the variable region
framework regions, preferably the third framework
region, leader region, promoter region, J region and
the like.

If the repertoires of V_H-coding and V_L-coding DNA
homologs are to be produced by (PCR) amplification,
two primers, i.e., a PCR primer pair, must be used for
each coding strand of nucleic acid to be amplified.
The first primer becomes part of the nonsense (minus
or complementary) strand and hybridizes to a
nucleotide sequence conserved among V_H (plus or
coding) strands within the repertoire. To produce V_H
coding DNA homologs, first primers are therefore
chosen to hybridize to (i.e. be complementary to)

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conserved regions within the J region, CH1 region, hinge region, CH2 region, or CH3 region of immunoglobulin genes and the like. To produce a V_L coding DNA homolog, first primers are chosen to
5   hybridize with (i.e. be complementary to) a conserved region within the J region or constant region of immunoglobulin light chain genes and the like. Second primers become part of the coding (plus) strand and hybridize to a nucleotide sequence conserved among
10 minus strands. To produce the V_H-coding DNA homologs, second primers are therefore chosen to hybridize with a conserved nucleotide sequence at the 5' end of the V_H-coding immunoglobulin gene such as in that area coding for the leader or first framework region. It
15 should be noted that in the amplification of both V_H- and V_L-coding DNA homologs the conserved 5' nucleotide sequence of the second primer can be complementary to a sequence exogenously added using terminal deoxynucleotidyl transferase as described by Loh et
20 al., Science, 243:217-220 (1989). One or both of the first and second primers can contain a nucleotide sequence defining an endonuclease recognition site. The site can be heterologous to the immunoglobulin gene being amplified and typically appears at or near
25 the 5' end of the primer.

When present, the restriction site-defining portion is typically located in a 5'-terminal non-priming portion of the primer. The restriction site defined by the first primer is typically chosen to be
30 one recognized by a restriction enzyme that does not recognize the restriction site defined by the second primer, the objective being to be able to produce a DNA molecule having cohesive termini that are non-complementary to each other and thus allow directional
35 insertion into a vector.

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In one embodiment, the present invention utilizes a set of polynucleotides that form primers having a priming region located at the 3'-terminus of the primer. The priming region is typically the 3'-most (3'-terminal) 15 to 30 nucleotide bases. The 3'-terminal priming portion of each primer is capable of acting as a primer to catalyze nucleic acid synthesis, i.e., initiate a primer extension reaction off its 3' terminus. One or both of the primers can additionally contain a 5'-terminal (5'-most) non-priming portion, i.e., a region that does not participate in hybridization to repertoire template.

In PCR, each primer works in combination with a second primer to amplify a target nucleic acid sequence. The choice of PCR primer pairs for use in PCR is governed by considerations as discussed herein for producing gene repertoires. That is, the primers have a nucleotide sequence that is complementary to a sequence conserved in the repertoire. Useful V_H and V_L priming sequences are shown in Tables 5 and 6, herein below.

4.   Polymerase Chain Reaction to Produce
      Gene Repertoires

The strategy used for cloning the V_H and V_L genes contained within a repertoire will depend, as is well known in the art, on the type, complexity, and purity of the nucleic acids making up the repertoire. Other factors include whether or not the genes are contained in one or a plurality of repertoires and whether or not they are to be amplified and/or mutagenized.

The V_H- and V_L-coding gene repertoires are comprised of polynucleotide coding strands, such as mRNA and/or the sense strand of genomic DNA. If the

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repertoire is in the form of double stranded genomic DNA, it is usually first denatured, typically by melting, into single strands. A repertoire is subjected to a PCR reaction by treating (contacting) the repertoire with a PCR primer pair, each member of the pair having a preselected nucleotide sequence. The PCR primer pair is capable of initiating primer extension reactions by hybridizing to nucleotide sequences, preferably at least about 10 nucleotides in length and more preferably at least about 20 nucleotides in length, conserved within the repertoire. The first primer of a PCR primer pair is sometimes referred to herein as the "sense primer" because it hybridizes to the coding or sense strand of a nucleic acid. In addition, the second primer of a PCR primer pair is sometimes referred to herein as the "anti-sense primer" because it hybridizes to a non-coding or anti-sense strand of a nucleic acid, i.e., a strand complementary to a coding strand.

The PCR reaction is performed by mixing the PCR primer pair, preferably a predetermined amount thereof, with the nucleic acids of the repertoire, preferably a predetermined amount thereof, in a PCR buffer to form a PCR reaction admixture. The admixture is maintained under polynucleotide synthesizing conditions for a time period, which is typically predetermined, sufficient for the formation of a PCR reaction product, thereby producing a plurality of different V_H-coding and/or V_L-coding DNA homologs.

A plurality of first primer and/or a plurality of second primers can be used in each amplification, e.g., one species of first primer can be paired with a number of different second primers to form several different primer pairs. Alternatively, an individual

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pair of first and second primers can be used. In any
case, the amplification products of amplifications
using the same or different combinations of first and
second primers can be combined to increase the
5   diversity of the gene library.
         In another strategy, the object is to clone the
V_H- and/or V_L-coding genes from a repertoire by
providing a polynucleotide complement of the
repertoire, such as the anti-sense strand of genomic
10   dsDNA or the polynucleotide produced by subjecting
mRNA to a reverse transcriptase reaction. Methods for
producing such complements are well known in the art.
         The PCR reaction is performed using any suitable
method. Generally it occurs in a buffered aqueous
15   solution, i.e., a PCR buffer, preferably at a pH of 7-
9, most preferably about 8. Preferably, a molar
excess (for genomic nucleic acid, usually about 10^6:1
primer:template) of the primer is admixed to the
buffer containing the template strand. A large molar
20   excess is preferred to improve the efficiency of the
process.
         The PCR buffer also contains the deoxyribo-
nucleotide triphosphates dATP, dCTP, dGTP, and dTTP
and a polymerase, typically thermostable, all in
25   adequate amounts for primer extension (polynucleotide
synthesis) reaction. The resulting solution (PCR
admixture) is heated to about 90°C - 100°C for about 1
to 10 minutes, preferably from 1 to 4 minutes. After
this heating period the solution is allowed to cool to
30   54°C, which is preferable for primer hybridization.
The synthesis reaction may occur at from room
temperature up to a temperature above which the
polymerase (inducing agent) no longer functions
efficiently. Thus, for example, if DNA polymerase is
35   used as inducing agent, the temperature is generally

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no greater than about 40°C. An exemplary PCR buffer
comprises the following: 50 mM KCl; 10 mM Tris-HCl;
pH 8.3; 1.5 mM $MgCl_2$; 0.001% (wt/vol) gelatin, 200 μM
dATP; 200 μM dTTP; 200 μM dCTP; 200 μM dGTP; and 2.5
5   units Thermus aquaticus DNA polymerase I (U.S. Patent
No. 4,889,818) per 100 microliters of buffer.
      The inducing agent may be any compound or system
which will function to accomplish the synthesis of
primer extension products, including enzymes.
10   Suitable enzymes for this purpose include, for
example, E. coli DNA polymerase I, Klenow fragment of
E. coli DNA polymerase I, T4 DNA polymerase, other
available DNA polymerases, reverse transcriptase, and
other enzymes, including heat-stable enzymes, which
15   will facilitate combination of the nucleotides in the
proper manner to form the primer extension products
which are complementary to each nucleic acid strand.
Generally, the synthesis will be initiated at the 3'
end of each primer and proceed in the 5' direction
20   along the template strand, until synthesis terminates,
producing molecules of different lengths. There may
be inducing agents, however, which initiate synthesis
at the 5' end and proceed in the above direction,
using the same process as described above.
25   The inducing agent also may be a compound or
system which will function to accomplish the synthesis
of RNA primer extension products, including enzymes.
In preferred embodiments, the inducing agent may be a
DNA-dependent RNA polymerase such as T7 RNA
30   polymerase, T3 RNA polymerase or SP6 RNA polymerase.
These polymerases produce a complementary RNA
polynucleotide. The high turn over rate of the RNA
polymerase amplifies the starting polynucleotide as
has been described by Chamberlin et al., The Enzymes,
35   ed. P. Boyer, PP. 87-108, Academic Press, New York

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(1982). Another advantage of T7 RNA polymerase is
that mutations can be introduced into the
polynucleotide synthesis by replacing a portion of
cDNA with one or more mutagenic oligodeoxynucleotides
(polynucleotides) and transcribing the partially-
mismatched template directly as has been previously
described by Joyce et al., Nuc. Acid Res., 17:711-722
(1989). Amplification systems based on transcription
have been described by Gingeras et al., in PCR
Protocols, A Guide to Methods and Applications, pp
245-252, Academic Press, Inc., San Diego, CA (1990).

If the inducing agent is a DNA-dependent RNA
polymerase and therefore incorporates ribonucleotide
triphosphates, sufficient amounts of ATP, CTP, GTP and
UTP are admixed to the primer extension reaction
admixture and the resulting solution is treated as
described above.

The newly synthesized strand and its
complementary nucleic acid strand form a double-
stranded molecule which can be used in the succeeding
steps of the process.

The first and/or second PCR reactions discussed
above can advantageously be used to incorporate into
the receptor a preselected epitope useful in
immunologically detecting and/or isolating a receptor.
This is accomplished by utilizing a first and/or
second polynucleotide synthesis primer or expression
vector to incorporate a predetermined amino acid
residue sequence into the amino acid residue sequence
of the receptor.

After producing V_H- and V_L-coding DNA homologs
for a plurality of different V_H- and V_L-coding genes
within the repertoires, the DNA molecules are
typically further amplified. While the DNA molecules
can be amplified by classic techniques such as

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incorporation into an autonomously replicating vector, it is preferred to first amplify the molecules by subjecting them to a polymerase chain reaction (PCR) prior to inserting them into a vector. PCR is

5   typically carried out by thermocycling i.e., repeatedly increasing and decreasing the temperature of a PCR reaction admixture within a temperature range whose lower limit is about 10°C to about 40°C and whose upper limit is about 90°C to about 100°C. The

10   increasing and decreasing can be continuous, but is preferably phasic with time periods of relative temperature stability at each of temperatures favoring polynucleotide synthesis, denaturation and hybridization.

15   PCR amplification methods are described in detail in U.S. Patent Nos. 4,683,195, 4,683,202, 4,800,159, and 4,965,188, and at least in several texts including "PCR Technology: Principles and Applications for DNA Amplification", H. Erlich, ed., Stockton Press, New

20   York (1989); and "PCR Protocols: A Guide to Methods and Applications", Innis et al., eds., Academic Press, San Diego, California (1990).

     In preferred embodiments only one pair of first and second primers is used per amplification reaction.

25   The amplification reaction products obtained from a plurality of different amplifications, each using a plurality of different primer pairs, are then combined.

     However, the present invention also contemplates

30   DNA homolog production via co-amplification (using two pairs of primers), and multiplex amplification (using up to about 8, 9 or 10 primer pairs).

     In preferred embodiments, the PCR process is used not only to produce a library of DNA molecules, but

35   also to induce mutations within the library or to

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create diversity from a single parental clone and
thereby provide a library having a greater
heterogeneity. First, it should be noted that the PCR
process itself is inherently mutagenic due to a

5   variety of factors well known in the art. Second, in
addition to the mutation inducing variations described
in the above referenced U.S. Patent No. 4,683,195,
other mutation inducing PCR variations can be
employed. For example, the PCR reaction admixture,

10 can be formed with different amounts of one or more of
the nucleotides to be incorporated into the extension
product. Under such conditions, the PCR reaction
proceeds to produce nucleotide substitutions within
the extension product as a result of the scarcity of a

15 particular base. Similarly, approximately equal molar
amounts of the nucleotides can be incorporated into
the initial PCR reaction admixture in an amount to
efficiently perform X number of cycles, and then
cycling the admixture through a number of cycles in

20 excess of X, such as, for instance, 2X.
Alternatively, mutations can be induced during the PCR
reaction by incorporating into the reaction admixture
nucleotide derivatives such as inosine, not normally
found in the nucleic acids of the repertoire being

25 amplified. During subsequent _in vivo_ DNA synthesis
and replication of the nucleic acids in a host cell,
the nucleotide derivative will be replaced with a
substitute nucleotide thereby inducing a point
mutation.

30

        5.   Linear DNA Expression Vectors
             A DNA expression vector for use in a
method of the invention for producing a library of DNA
molecules is a linearized DNA molecule as described

35 before having two (upstream and downstream) cohesive

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termini adapted for directional ligation to a
polypeptide gene.

A linear DNA expression vector is typically
prepared by restriction endonuclease digestion of a

5   circular DNA expression vector of this invention to
cut at two preselected restriction sites within the
sequence of nucleotides of the vector adapted for
directional ligation to produce a linear DNA molecule
having the required cohesive termini that are adapted

10   for direction ligation. Directional ligation refers
to the presence of two (a first and second) cohesive
termini on a vector, or on the insert DNA molecule to
be ligated into the vector selected, so that the
termini on a single molecule are not complementary. A

15   first terminus of the vector is complementary to a
first terminus of the insert, and the second terminus
of the vector is complementary to the second terminus
of the insert.

20   6.   Ligation Reactions to Produce Gene
                  Libraries
                  In preparing a library of DNA molecules
of this invention, a ligation admixture is prepared as
described above, and the admixture is subjected to

25   ligation conditions for a time period sufficient for
the admixed repertoire of polypeptide genes to ligate
(become operatively linked) to the plurality of DNA
expression vectors to form the library.
                  Ligation conditions are conditions selected to

30   favor a ligation reaction wherein a phosphodiester
bond is formed between adjacent 3' hydroxyl and 5'
phosporyl termini of DNA. The ligation reaction is
preferably catalyzed by the enzyme T4 DNA ligase.
Ligation conditions can vary in time, temperature,

35   concentration of buffers, quantities of DNA molecules

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to be ligated, and amounts of ligase, as is well
known. Preferred ligation conditions involve
maintaining the ligation admixture at 4 degrees
Centigrade (4°C) to 12°C for 1 to 24 hours in the
presence of 1 to 10 units of T4 DNA ligase per
milliliter (ml) and about 1 to 2 micrograms (ug) of
DNA. Ligation buffer in a ligation admixture
typically contains 0.5 M Tris-HCl (pH 7.4), 0.01 M
$MgCl_2$, 0.01 M dithiothrietol, 1 mM spermidine, 1 mM
ATP and 0.1 mg/ml bovine serum albumin (BSA). Other
ligation buffers can also be used.

Exemplary ligation reactions are described in
Example 2.

7.   Preparation of Dicistronic Gene
      Libraries

In a particularly preferred embodiment,
the present invention contemplates methods for the
preparation of a library of dicistronic DNA molecules.
A dicistronic DNA molecule is a single DNA molecule
having the capacity to express two separate
polypeptides from two separate cistrons. In preferred
embodiments, the two cistrons are operatively linked
at relative locations on the
DNA molecule such that both cistrons are under the
transcriptional control of a single promoter. Each
dicistronic molecule is capable of expressing first
and second polypeptides from first and second
cistrons, respectively, that can form, in a suitable
host, a heterodimeric receptor on the surface of a
filamentous phage particle.

The method for producing a library of dicistronic
DNA molecules comprises the steps of:

(a) Forming a first ligation admixture
by combining in a ligation buffer:

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          (i)   a repertoire of first
polypeptide genes in the form of dsDNA, each having
cohesive termini adapted for directional ligation, and
          (ii) a plurality of DNA expression
vectors in linear form, each having upstream and
downstream first cohesive termini that are. (a) adapted
for directionally receiving the first polypeptide
genes in a common reading frame, and (b) operatively
linked to respective upstream and downstream
translatable DNA sequences. The upstream translatable
DNA sequence encodes a pelB secretion signal, the
downstream translatable DNA sequence encodes a
filamentous phage coat protein membrane anchor, and
translatable DNA sequences are operatively linked to
respective upstream and downstream DNA expression
control sequences.

          (b)   Subjecting the admixture to
ligation conditions for a time period sufficient to
operatively link the first polypeptide genes to the
vectors and produce a plurality of circular DNA
molecules each having a first cistron for expressing
the first polypeptide.

          (c)   Treating the plurality of circular
DNA molecules under DNA cleavage conditions to produce
a plurality of DNA expression vectors in linear form
that each have upstream and downstream second cohesive
termini that are (i) adapted for directionally
receiving a repertoire of second polypeptide genes in
a common reading frame, and (ii) operatively linked to
respective upstream and downstream DNA sequences. The
upstream DNA sequence is a translatable sequence
encoding a secretion signal, the downstream DNA
sequence has at least one stop codon in the reading
frame, and the translatable DNA sequence is
operatively linked to a DNA expression control

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sequence.

(d) Forming a second ligation admixture by combining in a ligation buffer:

(i) the plurality of DNA expression vectors formed in step (c), and

(ii) the repertoire of second polypeptide genes in the form of dsDNA, each having cohesive termini adapted for directional ligation to the plurality of DNA expression vectors; and

(e) Subjecting the second admixture to ligation conditions for a time period sufficient to operatively link the second polypeptide genes to said vectors and produce a plurality of circular DNA molecules each having the second cistron for expressing the second polypeptide, thereby forming the library.

In preferred embodiments a secretion signal is a pelB secretion signal. Also preferred is the use of a filamentous phage membrane anchor that is derived from cpIII or cpVIII as described herein.

DNA expression vectors useful for practicing the above method are the dicistronic expression vectors described in greater detail before.

In practicing the method of producing a library of dicistronic DNA molecules, it is preferred that the upstream and downstream first cohesive termini do not have the same nucleotide sequences as the upstream and downstream second cohesive termini. In this embodiment, the treating step (c) to linearize the circular DNA molecules typically involves the use of restriction endonucleases that are specific for producing said second termini, but do not cleave the circular DNA molecule at the sites that formed the first termini. Exemplary and preferred first and second termini are the termini defined by cleavage of

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pCBAK8 with Xho I and Spe I to form the upstream and
downstream first termini, and defined by cleavage of
pCBAK8 with Sac I and Xba I to form the upstream and
downstream second termini. In this embodiment, other
5   pairs of cohesive termini can be utilized at the
respective pairs of first and second termini, so long
as the four termini are each distinct, non-
complementary termini. Exemplary are the termini
found on the vectors pCOMB3, pCOMB2-3, pCOMB2-3',
10   pCOMB8 and pCOMB2-8 described herein.

Methods of treating the plurality of circular DNA
molecules under DNA cleavage conditions to form linear
DNA molecules are generally well known and depend on
the nucleotide sequence to be cleaved and the
15   mechanism for cleavage. Preferred treatments involve
admixing the DNA molecules with a restriction
endonuclease specific for a endonuclease recognition
site at the desired cleavage location in an amount
sufficient for the restriction endonuclease to cleave
20   the DNA molecule. Buffers, cleavage conditions, and
substrate concentrations for restriction endonuclease
cleavage are well known and depend on the particular
enzyme utilized. Exemplary restriction enzyme
cleavage conditions are described in Example 2.

25   In a related embodiment, the invention provides a
method for producing a library of DNA molecules having
a single cistron, following the method described
previously and stopping after completing step (b).
Such a library contains DNA molecules each comprising
30   a cistron for expressing a polypeptide of this
invention.

8.   Methods for Changing the Diversity of a
        Library
35   The present invention provides methods

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for changing the diversity of a library of filamentous
phage library of this invention. These methods
generally increase the diversity of the library,
thereby increasing the pool of possible epitope-
5   binding complexes from which to screen for a desired
binding activity. Alternatively, the methods can be
directed at enriching for a class of epitope-binding
complexes. The class is typically defined by the
ability to bind a particular epitope or family of .
10   epitopes present on a preselected antigen or group of
antigens.

                    a.   Increasing Library Diversity by
                            Mutation
15   A particularly preferred method
for increasing diversity is to alter the amino acid
residue sequence of one or more polypeptides of the
epitope-binding complex encoded by the genome of a
phage of this invention. Alterations can be
20   conveniently introduced at the nucleic acid level by
mutation of the nucleic acid. The method can be
practiced on a single species of nucleic acid coding a
polypeptide of this invention, or can be practiced on
a library of nucleic acids present in a library of
25   phage of this invention.
        Mutation of nucleic acid can be conducted by a
variety of means, but is most conveniently conducted
in a PCR reaction during a PCR process of the present
invention. PCR mutagenesis can be random or directed
30   to specific nucleotide sequences, as is generally well
known. Conducting PCR under conditions favorable to
random mutagenesis has been described previously, and
is referred to as "error prone PCR". Similarly,
directed mutagenesis involves the use of PCR primers
35   designed to target a specific type of mutation into a

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specific region of nucleotide sequence.

In one embodiment, the invention contemplates increasing diversity of one or more epitope-binding complexes by PCR-directed mutation of a complementarity determining region (CDR) of an antibody variable domain present in an epitope-binding complex polypeptide of this invention. CDR mutagenesis has been previously described in general terms for "humanizing" an antibody by introducing human sequences into the CDR region of a murine antibody. See European Application No. EP 239400.

Thus the invention contemplates a mutagenesis method for altering the immunological specificity of a cloned immunoglobulin gene present in a DNA vector of this invention. The method provides directed mutagenesis in a preselected CDR of an immunoglobulin gene which comprises subjecting a recombinant DNA molecule (rDNA) containing the cloned immunoglobulin gene having a target CDR to PCR conditions suitable for amplifying a preselected region of the CDR. In the method, the rDNA molecule is subjected to PCR conditions that include a PCR primer oligonucleotide as described below constituting the first primer in a PCR primer pair as is well known to produce an amplified PCR product that is derived from the preselected CDR region but that includes the nucleotide sequences of the PCR primer. The second oligonucleotide in the PCR amplifying conditions can be any PCR primer derived from the immunoglobulin gene to be mutagenized, as described herein.

Preferred are methods using an oligonucleotide of this invention as described below.

In a related embodiment, therefore, an oligonucleotide is contemplated that is useful as a primer in a polymerase chain reaction (PCR) for

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inducing mutagenesis in a complementarity determining
region (CDR) of an immunoglobulin gene. The
oligonucleotide has 3' and 5' termini and comprises
(1) a nucleotide sequence at its 3' terminus capable
5   of hybridizing to a first framework region of an
immunoglobulin gene, (2) a nucleotide sequence at its
5' terminus capable of hybridizing to a second
framework region of an immunoglobulin gene, and (3) a
nucleotide sequence between the 3' and 5' termini
10   adapted for introducing mutations during a PCR into
the CDR region between the first and second framework
regions of the immunoglobulin gene, thereby
mutagenizing the CDR region.

Insofar as immunoglobulin genes have three CDR
15   regions on both the heavy chain and the light chain of
an immunoglobulin, each separated by a distinctive
framework region, it is to be understood that the
above example is readily applicable to introducing
mutations into a specific CDR by selection of the
20   above 5' and 3' nucleotide sequences as to hybridize
to the framework regions flanking the targeted CDR.
Thus the above first and second framework sequences
can be the conserved sequences flanking CDR1, CDR2 or
CDR3 on either the heavy or light chain. Exemplary
25   and preferred is the CDR3 of the human immunoglobulin
heavy chain.

The length of the 3' and 5' terminal nucleotide
sequences of a subject mutagenizing oligonucleotide
can vary in length as is well known, so long as the
30   length provides a stretch of nucleotides complementary
to the target framework sequences as to hybridize
thereto. In the case of the 3' terminal nucleotide
sequence, it must be of sufficient length and
complementarity to the target framework region located
35   3' to the CDR region to be mutagenized as to hybridize

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and provide a 3'hydroxyl terminus for initiating a
primer extension reaction. In the case of the 5'
terminal nucleotide sequence, it must be of sufficient
length and complementarity to the target framework
5   region located 5' to the CDR region to be mutagenized
as to provide a means for hybridizing in a.PCR overlap
extension reaction as described above to assemble the
complete immunoglobulin heavy or light chain.

     Framework regions flanking a CDR are well
10   characterized in the immunological arts, and include
known nucleotide sequences or consensus sequences as
described elsewhere herein. Where a single,
preselected immunoglobulin gene is to be mutagenized,
the framework-defined sequences flanking a particular
15   CDR are known, or can be readily determined by
nucleotide sequencing protocols. Where a repertoire
of immunoglobulin genes are to be mutagenized, the
framework-derived sequences are preferably conserved,
as described elsewhere herein.

20   Preferably, the length of the 3' and 5' terminal
nucleotide sequences are each at least 6 nucleotides
in length, and can be up to 50 or more nucleotides in
length, although these lengths are unnecessary to
assure accurate and reproducible hybridization.
25   Preferred are lengths in the range of 12 to 30
nucleotides, and typically are about 18 nucleotides.

     A particularly preferred framework-defined
nucleotide sequence for use as a 3' terminus
nucleotide sequence has the nucleotide sequence 5'-
30   TGGGGCCAAGGGACCACG-3' (SEQ ID NO 122).

     A particularly preferred framework-defined
nucleotide sequence for use as a 5' terminus
nucleotide sequence has the nucleotide sequence 5'-
GTGTATTATTGTGCGAGA-3' (SEQ ID NO 123).
35   The nucleotide sequence located between the 3'

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and 5' termini adapted for mutagenizing a CDR can be
any nucleotide sequence, insofar as the novel sequence
will be incorporated by the above methods. However,
the present approach provides a means to produce a
large population of mutagenized CDR's in a single PCR
reaction by the use of a population of redundant
sequences defining randomized or nearly randomized
nucleotides in the CDR region to be mutagenized.

A preferred oligonucleotide comprises a
nucleotide sequence between the above described 3' and
5' termini that is represented by the formula:
$[NNR]_n$, wherein N can independently be any
nucleotide, R can be S, K or analogs thereof, where S
is G or C, K is G or T, and where n is from 3 to about
24. In preferred embodiments, the oligonucleotide has
the formula:
5'-GTGTATTATTGTGCGAGA$[NNR]_n$TGGGGCCAAGGGACCACG-3' (SEQ
ID NO 124).
Exemplary and particularly preferred is the
oligonucleotide where R is S and n is 16, such that
the oligonucleotide represents a large population of
redundant oligonucleotide sequences.

Thus, the invention contemplates a method for
increasing the diversity of a library of filamentous
phage particles comprising the steps of: a) providing
a library of filamentous phage particles according to
the present invention, and b) mutating the
immunoglobulin variable domain-coding nucleotide
sequence present in each DNA expression vector in the
library to form a library of phage particles each
containing a mutated immunoglobulin variable domain
nucleotide sequence.

The providing can include manipulating the
genomes of the phage particles in the library in order
to isolate the nucleic acids in preparation for a

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mutagenizing PCR reaction. Manipulations of a phage
library to isolate the phage genome for use in a PCR
reaction is described elsewhere herein.

In one embodiment, the mutating comprises
5   subjecting the immunoglobulin variable domain-coding
nucleotide sequence to an error-prone polymerase chain
reaction. In another embodiment, the mutating
comprises subjecting the immunoglobulin variable
domain-coding nucleotide sequence to a method for
10   mutating a CDR of the immunoglobulin variable domain-
coding nucleotide sequence using a CDR-directed
oligonucleotide as described herein.

Exemplary methods of mutating the CDR region of a
particular epitope-binding complex coding nucleic acid
15   using the above CDR-directing oligonucleotide or using
error-prone PCR to produce a large library of diverse
complexes is described in the Examples.

b.   Enrichment of a Library
20   The invention describes a method to
change the diversity of the library by enriching the
library for a preselected class of epitope-binding
complexes. The process generally involves affinity
selection of those phage particles in a library that
25   are capable of binding a preselected antigen. The
process of affinity selection, or panning, is
described in detail in the Examples.

Thus the invention contemplates a method for
changing the diversity of a library of filamentous
30   phage particles comprising the steps of a) providing a
library of filamentous phage particles according to
the present invention, b) contacting the provided
library with a preselected ligand under conditions
sufficient for members of the library to bind to the
35   ligand and form a ligand-phage particle complex, and

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c) isolating phage particles in the complex away from
non-bound library members to form a ligand-enriched
library comprising phage particles having binding
specificity for the preselected ligand.

5   In preferred embodiments, the preselected ligand
is affixed to a solid support, and the ligand-phage
particle complex is formed in the solid phase. This
embodiment further comprises the steps of i) washing
the solid support after the contacting step to rinse

10   non-bound library members from the solid support; and
ii) eluting any solid-phase bound phage particles off
of the solid support. The eluted phage particles are
collected, thereby forming isolated phage particles
that comprise an enriched library.

15   Elution can be conducted under a variety of
conditions that disrupt the ligand-epitope-binding
complex interaction. Typical conditions include high
salt or low pH buffers. Particularly preferred are
buffers of about pH 1 to 5, preferably about pH 2 to

20   3. Alternatively, the interaction can be disrupted by
competition with an excess amount of the preselected
ligand in the elution buffer. Both elution procedures
are described in the Examples.

           A related embodiment combines the features of

25   both increasing diversity of a library by mutation and
enriching the library by panning to "mature" epitope-
binding complex affinities for a preselected ligand.
Thus it is possible to evolve new binding
specificities, and more potent binding specificities,

30   using the present methods for changing library
diversity.

           The combination of these methods can be
configured in a variety of ways, as will be apparent
to a skilled practitioner. For example, one can

35   isolate a library, mutagenize (diversify), and then

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screen (enrich) for a particular binding activity.
Alternatively, one can enrich for a particular
activity from a library, mutagenize the specific
epitope-binding complex and further enrich the library
5   produced by the mutagenesis.

In another permutation on this theme, one can
utilize the differences between libraries based on
cpIII- and cpVIII-derived membrane anchors due to
their inherent differences in valency. Because a
10   library of phage having the cpIII-derived membrane
anchor will typically contain only 1 to 4 copies of
the epitope-binding complex on the surface of each
phage particle, the phage presents a binding complex
of relatively "low" valency, approaching one. In
15   contrast, a library of phage having a cpVIII-derived
membrane anchor will typically contain 20 to 1000
copies of the epitope-binding complex on the surface
of each phage particle, the particle presents a
relatively "high" valency. Thus, cpIII-based
20   libraries are referred to as monovalent and cpVIII-
based libraries are referred to as multivalent.

Applying the well-known principles of antibody
affinity and valence, it is understood that a cpIII-
based library can be enriched upon screening for
25   generally higher affinity binding interactions
(binding constants of 10^6 to 10^9 M^{-1}) as compared to
the broader range of affinities (binding constants of
10^4 to 10^9 M^{-1}) isolatable using a multivalent reagent
found in the cpVIII-based library. Therefore, a
30   cpVIII-based library is useful to isolate a broad
range of affinities of epitope-binding complexes from
low to high, whereas a cpIII-based library is useful
to isolate a narrower range of higher affinity
epitope-binding complexes.
35   Thus the invention contemplates producing a first

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enriched library by enrichment of a cpVIII-based
library. Thereafter the genes for encoding the
epitope-binding complex polypeptides are transferred
into a cpIII-based vector, and subsequently enriched
5   for a high affinity binding interaction. In one
embodiment, a mutation step can be utilized prior to
the transfer into the cpIII-based vector.
       In another embodiment, the ability to mature a
novel affinity is shown by an example herein in which
10   a cloned V_H/V_L heterodimer-coding gene capable of
expressing a heterodimer that binds the ligand tetanus
toxoid (TT) is mutagenized using CDR-directed PCR
mutagenesis, and the mutagenized nucleic acid
population resulting therefrom is inserted into a
15   cpIII-based library and screened for binding to a
different ligand, fluorescein. A high affinity
epitope-binding complex was identified that binds
fluorescein.
       In a related embodiment, a naive (non-immunized)
20   library was cloned into a cpVIII-based library and
screened for binding to the antigen progesterone. Low
affinity binders were cloned into a cpIII-based
library, and three high affinity binding clones
identified that bind progesterone. The three clones
25   were pooled, and the pool was subjected to error-prone
PCR mutagenesis, and the resulting library of mutated
nucleic acids were cloned into a cpIII-based vector
and screened against progesterone to yield a high
affinity epitope-binding complex that binds
30   progesterone. Thus a high affinity complex was
"matured" from a naive library.
       Thus, the present invention also contemplates a
method for maturing the affinity of an epitope-binding
complex encoded by a filamemtous phage of this
35   invention comprising the steps of a) providing the

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genome of a filamentous phage, b) mutating the
immunoglobulin variable domain-coding nucleotide
sequence present in the provided genome to form a
library of phage particles containing a mutated
5   immunoglobin variable domain nucleotide sequence, c)
contacting the library formed in step (b) ·with a
preselected ligand under conditions sufficient for
members of the library to bind to the ligand and form
a ligand-phage particle complex, and d) isolating ·
10   phage particles in said complex away from non-bound
library members to form a ligand-enriched library
comprising phage particles having binding specificity
for the preselected ligand.

15   F.   Phage Libraries
            The present invention contemplates a library
of DNA molecules that each encode a fusion polypeptide
of this invention where the library is in the form of
a population of different filamentous phage particles
20   each containing a different rDNA molecule of this
invention.   By different rDNA molecule is meant a rDNA
molecule differing in nucleotide base sequence
encoding a polypeptide of this invention when compared
in nucleotide sequence to another rDNA molecule in the
25   library.
            Thus, a phage library is a population of
filamentous phage, preferably fl, fd or M13
filamentous phage, each phage having packaged inside
the particle a rDNA expression vector of this
30   invention, the rDNA is encapsulated in the phage
particle by the matrix proteins of the phage. Stated
differently, a phage library contains a plurality of
filamentous phage particles, each different phage
particle containing at least one epitope-binding
35   complex on its surface as described herein. A

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preferred library is comprised of phage particles
containing DNA molecules that encode at least 10^6,
preferably 10^7 and more preferably 10^{8-9} different
fusion polypeptides of this invention. By different
fusion polypeptides is meant fusion polypeptides
differing in amino acid residue sequence. Even higher
library diversities are available when the methods of
random combination or mutagenesis are utilized as
described herein to increase library diversity.

Where the packaged expression vector encodes
first and second polypeptides of an autogenously
assembling receptor, e.g. V_H and V_L polypeptides that
form a Fab, the library can also be characterized as
containing or expressing a multiplicity of receptor
specificities. Thus, libraries express at least 10^5,
preferably at least 10^6 and more preferably at least
10^7 different receptors, such as different antibodies,
T cell receptors, integrins and the like.

The size of the library can vary depending on a
number of factors, particularly the method in which
the library is produced. As used herein, size
connotes the complexity or diversity of the library,
that is the number of different species making up the
library, rather than the absolute number of particles
in the library.

Thus, where a library is produced by first
separately cloning two repertoires of genes,
corresponding to the first and second polypeptides,
the resulting library size after randomly combining
the two repertoires in the form of a dicistronic
vector is greatly increased. For example, consider
light chain and heavy chain variable antibody gene
repertoires, each having 10^6 different members.
Combining the two repertoires theoretically yields a
library of 10^{12} possible different dicistronic vector

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species.

An experimental system was designed to evaluate
the ability to "shuffle" two repertoires as described
above in order to generate greater diversity. The

5   system utilized a combinatorial Fab library derived
from a mouse immunized with the hapten para-
nitrophenyl phosphonamidate (NPN). Twenty-two
different clones were isolated, and the heavy and
light chain coding nucleic acids were isolated and

10   sequenced to determine that 21 of the 22 pairs were
different at the level of nucleic acid sequence. The
22 NPN ligand-binding clones were randomly recombined
(shuffled), and rescreened for binding to NPN.

Assuming that the heavy and light chains can only

15   form ligand-binding heterodimeric receptor molecules
if the original pairs are rejoined, the model predicts
that 4.6 percent of the total combinations would
provide ligand-binding combinations. Any higher
percentage demonstrates that pairings other than the

20   original pairs are also capable of binding NPN. The
results showed that 27 percent of the clones isolated
bound NPN, indicating a 5.8-fold increase in the
library size of receptors able to bind NPN upon
shuffling. This demonstrated increase is limited to

25   those clones that bind NPN. Other members of the
randomly shuffled library have the capacity to bind
diverse, non-NPN, ligands. Thus, shuffling was shown
to increase diversity.

Library complexity can also be increased using

30   the methods herein for mutating nucleotide sequences
in a pre-existing library of sequences. Stated in
terms of amino acid residue differences for an
expressed fusion polypeptide, there can be potentially
a twenty-fold increase in library size for each amino

35   acid residue position that is targeted for random

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mutation.

For example, using the complementarity determining region (CDR)-directed mutagenesis of antibody genes as described in the Examples, a linear region of 16 amino acid residues was targeted for random mutation. Starting with a single species and mutating all 16 residue positions through all possible combinations with a choice of 20 different amino acids would theoretically produce a library of 20^{16} different species, or 6×10^{20} different species.

As described herein, a particular advantage of a filamentous phage in the present invention is that the DNA molecule present in the phage particle and encoding one or both of the members of the heterodimeric receptor can be segregated from other DNA molecules present in the library on the basis of the presence of the particular expressed fusion polypeptide the surface of the phage particle.

Isolation (segregation) of a DNA molecule encoding one or both members of a heterodimeric receptor is conducted by segregation of the filamentous phage particle containing the gene or genes of interest away from the population of other phage particles comprising the library. Segregation of phage particles involves the physical separation and propagation of individual phage particles away from other particles in the library. Methods for physical separation of filamentous phage particles to produce individual particles, and the propagation of the individual particles to form populations of progeny phage derived from the individual segregated particle are well known in the filamentous phage arts.

A preferred separation method involves the identification of the expressed heterodimer on the surface of the phage particle by means of a ligand

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binding specificity between the phage particle and a preselected ligand. Exemplary and preferred is the use of "panning" methods whereby a suspension of phage particles is contacted with a solid phase ligand (antigen) and allowed to specifically bind (or immunoreact where the heterodimer includes an immunoglobulin variable domain). After binding, non-bound particles are washed off the solid phase, and the bound phage particles are those that contain ligand-specific heterodimeric receptor (heterodimer) on their surface. The bound particles can then be recovered by elution of the bound particle from the solid phase, typically by the use of aqueous solvents that interfere with the ligand-receptor interaction. Typical solvent include buffers having high ionic strength, low pH, or an amount of soluble competing ligand sufficient to disrupt the receptor-ligand binding interaction.

An alternate method for separating a phage particle based on the ligand specificity of the surface-expressed heterodimer from a population of particles is to precipitate the phage particles from the solution phase by crosslinkage with the ligand. An exemplary and preferred crosslinking and precipitation method is described in detail in Example 4c.

The use of the above particle segregation methods provides a means for screening a population of filamentous phage particles present in a phage library of this invention. As applied to a phage library, screening can be utilized to enrich the library for one or more particles that express a heterodimer having a preselected ligand binding specificity. Where the library is designed to contain multiple species of heterodimers that all have some detectable

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measure of ligand binding activity, but differ in
protein structure, antigenicity, ligand binding
affinity or avidity, and the like, the screening
methods can be utilized sequentially to first produce

5   a library enriched for a preselected binding
specificity, and then to produce a second library
further enriched by further screening comprising one
or more isolated phage particles. Methods for
measuring ligand binding activities, antigenicity and

10   the like interactions between a ligand and a receptor
are generally well known and are not discussed further
as they are not essential features of the present
invention.

Thus, in one embodiment, a phage library is a

15   population of particles enriched for a preselected
ligand binding specificity.

In another embodiment, a phage library comprises
a population of particles wherein each particle
contains at least one fusion polypeptide of this

20   invention on the surface of the phage particle. The
actual amount of fusion polypeptide present on the
surface of a phage particle depends, in part, on the
choice of coat protein membrane anchor present in the
fusion polypeptide.

25   Where the anchor is derived from cpIII, there are
typically about 1 to 4 fusion polypeptides per phage
particle. Where the anchor is derived from the more
preferred cpVIII, there is the potential for hundreds
of fusion polypeptides on the particle surface

30   depending on the growth conditions and other factors
as discussed herein. The actual amount of fusion
polypeptides present on a phage particle can be
adjusted by controlling the amount "captured" by the
phage particle as it is being synthesized in a host

35   cell.

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Typically, a phage particle in a library of this invention contains from about 10 to about 500 cpVIII-derived fusion polypeptides on the surface of each particle, and more preferably about 20 to 50 fusion polypeptides per particle. Exemplary amounts of surface fusion polypeptide are shown by the electron micrographs described in Example 4a that describe particles having about 20 to 24 cpVIII-derived fusion polypeptides per particle.

In another embodiment, the present invention contemplates a population of phage particles that are the progeny of a single particle, and therefor all express the same heterodimer on the particle surface. Such a population of phage are homogeneous and clonally derived, and therefore provide a source for expressing large quantities of a particular fusion polypeptide. An exemplary clonally homogeneous phage population is described in Example 4.

A filamentous phage particle in a library of this invention is produced by standard filamentous phage particle preparation methods and depends on the presence in a DNA expression vector of this invention of a filamentous phage origin of replication as described herein to provide the signals necessary for (1) production of a single-stranded filamentous phage replicative form and (2) packaging of the replicative form into a filamentous phage particle. Such a DNA molecule can be packaged when present in a bacterial cell host upon introduction of genetic complementation to provide the filamentous phage proteins required for production of infectious phage particles. A typical and preferred method for genetic complementation is to infect a bacterial host cell containing a DNA expression vector of this invention with a helper filamentous phage, thereby providing the genetic

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elements required for phage particle assembly.
Exemplary helper rescue methods are described herein
at Example 2, and described by Short et al., <u>Nuc.</u>
<u>Acids Res.</u>, 16:7583-7600 (1988).

5   The level of heterodimeric receptor captured on
the surface of a filamentous phage particle during the
process of phage particle extrusion from the host cell
can be controlled by a variety of means. In one
embodiment, the levels of fusion polypeptides are

10   controlled by the use of strong promoters in the first
and second cistrons for expressing the polypeptides,
such that transcription of the fusion polypeptide
cistrons occurs at a relative rate greater than the
rate of transcription of the cpVIII gene on the helper

15   phage. In another embodiment, the helper phage can
have an amber mutation in the gene for expressing
cpVIII, such that less wild-type cpVIII is transcribed
in the host cell than fusion polypeptides, thereby
leading to increased ratios of fusion polypeptide

20   compared to cpVIII during the extrusion process.

In another embodiment, the amount of
heterodimeric receptor on the phage particle surface
can be controlled by controlling the timing between
expression of fusion polypeptides and the

25   superinfection by helper phage. After introduction of
the expression vector into the host cell, longer delay
times before the addition of helper phage will allow
for increased accumulation of the fusion polypeptides
in the host cell, thereby increasing the amount of

30   fusion polypeptide captured by the extruding phage
particle.

         G.   <u>Diagnostic Methods</u>
               The present invention also describes a
35   diagnostic system, preferably in kit form, for

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assaying for the presence of a preselected ligand, or
antigen, in a sample where it is desirable to detect
the presence, and preferably the amount, of the ligand
or antigen in a sample according to the diagnostic

5   methods described herein.

The sample can be a tissue, tissue extract, fluid
sample or body fluid sample, such as blood, plasma or
serum.

The diagnostic system includes, in an amount

10   sufficient to perform at least one assay, a
filamentous phage or ligand-binding heterodimeric
receptor according to the present invention, as a
separately packaged reagent.

Exemplary diagnostic systems for detecting a

15   preselected ligand in the solid phase and utilizing a
filamentous phage of this invention are described in
the Examples.

Instructions for use of the packaged reagent(s)
are also typically included.

20   As used herein, the term "package" refers to a
solid matrix or material such as glass, plastic (e.g.,
polyethylene, polypropylene or polycarbonate), paper,
foil and the like capable of holding within fixed
limits a heterodimeric receptor, filamentous phage or

25   library of phage of the present invention. Thus, for
example, a package can be a glass vial used to contain
milligram quantities of a contemplated labeled phage
preparation, or it can be a microtiter plate well to
which microgram quantities of a contemplated receptor

30   or phage particle(s) have been operatively affixed,
i.e., linked so as to be capable of binding a ligand.

"Instructions for use" typically include a
tangible expression describing the reagent
concentration or at least one assay method parameter

35   such as the relative amounts of reagent and sample to

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be admixed, maintenance time periods for reagent/
sample admixtures, temperature, buffer conditions and
the like.

A diagnostic system of the present invention
5   preferably also includes a label or indicating means
capable of signaling the formation of a binding
reaction complex containing a ligand-binding
heterodimeric receptor or phage complexed with the
preselected ligand.

10   The word "complex" as used herein refers to the
product of a specific binding reaction such as an
phage-ligand or receptor-ligand reaction. Exemplary
complexes are immunoreaction products.

As used herein, the terms "label" and "indicating
15   means" in their various grammatical forms refer to
single atoms and molecules that are either directly or
indirectly involved in the production of a detectable
signal to indicate the presence of a complex. Any
label or indicating means can be linked to or
20   incorporated in an expressed polypeptide, or phage
particle that is used in a diagnostic method. Such
labels are themselves well-known in clinical
diagnostic chemistry and constitute a part of this
invention only insofar as they are utilized with
25   otherwise novel proteins methods and/or systems.

The labeling means can be a fluorescent labeling
agent that chemically binds to antibodies or antigens
without denaturing them to form a fluorochrome (dye)
that is a useful immunofluorescent tracer. Suitable
30   fluorescent labeling agents are fluorochromes such as
fluorescein isocyanate (FIC), fluorescein
isothiocyante (FITC), 5-dimethylamine-1-
naphthalenesulfonyl chloride (DANSC),
tetramethylrhodamine isothiocyanate (TRITC),
35   lissamine, rhodamine 8200 sulphonyl chloride (RB 200

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SC) and the like. A description of immunofluorescence
analysis techniques is found in DeLuca,
"Immunofluorescence Analysis", in Antibody As a Tool,
Marchalonis, et al., eds., John Wiley & Sons, Ltd.,
5   pp. 189-231 (1982), which is incorporated herein by
reference.

In preferred embodiments, the indicating group is
an enzyme, such as horseradish peroxidase (HRP),
glucose oxidase, or the like. In such cases where the
10   principal indicating group is an enzyme such as HRP or
glucose oxidase, additional reagents are required to
visualize the fact that a receptor-ligand complex
(immunoreactant) has formed. Such additional reagents
for HRP include hydrogen peroxide and an oxidation dye
15   precursor such as diaminobenzidine. An additional
reagent useful with glucose oxidase is 2,2'-amino-di-
(3-ethyl-benzthiazoline-G-sulfonic acid) (ABTS).

Radioactive elements are also useful labeling
agents and are used illustratively herein. An
20   exemplary radiolabeling agent is a radioactive element
that produces gamma ray emissions. Elements which
themselves emit gamma rays, such as ^{124}I, ^{125}I, ^{128}I, ^{132}I
and ^{51}Cr represent one class of gamma ray emission-
producing radioactive element indicating groups.
25   Particularly preferred is ^{125}I. Another group of
useful labeling means are those elements such as ^{11}C,
^{18}F, ^{15}O and ^{13}N which themselves emit positrons. The
positrons so emitted produce gamma rays upon
encounters with electrons present in the animal's
30   body. Also useful is a beta emitter, such ^{111} indium
of ^{3}H.

. The linking of labels, i.e., labeling of,
polypeptides and proteins is well known in the art.
For instance, proteins or phage can be labeled by
35   metabolic incorporation of radioisotope-containing

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amino acids provided as a component in the culture
medium. See, for example, Galfre et al., Meth.
Enzymol., 73:3-46 (1981). The techniques of protein
conjugation or coupling through activated functional
5   groups are particularly applicable. See, for example,
Aurameas, et al., Scand. J. Immunol., Vol. 8 Suppl.
7:7-23 (1978), Rodwell et al., Biotech., 3:889-894
(1984), and U.S. Pat. No. 4,493,795.

        The diagnostic systems can also include,
10   preferably as a separate package, a specific binding
agent. A "specific binding agent" is a molecular
entity capable of selectively binding a reagent
species of the present invention or a complex
containing such a species, but is not itself a
15   polypeptide or phage of the present invention.
Exemplary specific binding agents are antibody
molecules, complement proteins or fragments thereof,
S. aureus protein A, and the like. Preferably the
specific binding agent binds the reagent species when
20   that species is present as part of a complex.

        In preferred embodiments, the specific binding
agent is labeled. However, when the diagnostic system
includes a specific binding agent that is not labeled,
the agent is typically used as an amplifying means or
25   reagent. In these embodiments, the labeled specific
binding agent is capable of specifically binding the
amplifying means when the amplifying means is bound to
a reagent species-containing complex.

        The diagnostic kits of the present invention can
30   be used in an "ELISA" format to detect the quantity of
a preselected ligand in a fluid sample. "ELISA"
refers to an enzyme-linked immunosorbent assay that
employs an antibody or antigen bound to a solid phase
and an enzyme-antigen or enzyme-antibody conjugate to
35   detect and quantify the amount of an antigen present

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in a sample and is readily applicable to the present
methods. A description of the ELISA technique is
found in Chapter 22 of the 4th Edition of <u>Basic and
Clinical Immunology</u> by D.P. Sites et al., published by
Lange Medical Publications of Los Altos, CA in 1982
and in U.S. Patents No. 3,654,090; No. 3,850,752; and
No. 4,016,043, which are all incorporated herein by
reference.

Thus, in some embodiments, a polypeptide, or a
phage of the present invention can be affixed to a
solid matrix to form a solid support that comprises a
package in the subject diagnostic systems.

A reagent is typically affixed to a solid matrix
by adsorption from an aqueous medium although other
modes of affixation applicable to proteins and
polypeptides can be used that are well known to those
skilled in the art. Exemplary adsorption methods are
described herein.

Useful solid matrices are also well known in the
art. Such materials are water insoluble and include
the cross-linked dextran available under the trademark
SEPHADEX from Pharmacia Fine Chemicals (Piscataway,
NJ); agarose; beads of polystyrene beads about 1
micron to about 5 millimeters in diameter available
from Abbott Laboratories of North Chicago, IL;
polyvinyl chloride, polystyrene, cross-linked
polyacrylamide, nitrocellulose- or nylon-based webs
such as sheets, strips or paddles; or tubes, plates or
the wells of a microtiter plate such as those made
from polystyrene or polyvinylchloride.

The reagent species, labeled specific binding
agent or amplifying reagent of any diagnostic system
described herein can be provided in solution, as a
liquid dispersion or as a substantially dry power,
e.g., in lyophilized form. Where the indicating means

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is an enzyme, the enzyme's substrate can also be
provided in a separate package of a system. A solid
support such as the before-described microtiter plate
and one or more buffers can also be included as
5   separately packaged elements in this diagnostic assay
system.

       The packaging materials discussed herein in
relation to diagnostic systems are those customarily
utilized in diagnostic systems.
10

       H.   Assay Methods
              The present invention contemplates various
assay methods for determining the presence, and
preferably amount, of a preselected ligand, typically
15   present in an aqueous composition such as a biological
fluid sample using a heterodimeric receptor or phage
of this invention as an ligand-binding reagent to form
a binding reaction product whose amount relates,
either directly or indirectly, to the amount of the
20   preselected ligand in the sample.

       Those skilled in the art will understand that
there are numerous well known clinical diagnostic
chemistry procedures in which a binding reagent of
this invention can be used to form an binding reaction
25   product whose amount relates to the amount of the
ligand in a sample. Thus, while exemplary assay
methods are described herein, the invention is not so
limited.

       Various heterogenous and homogeneous protocols,
30   either competitive or noncompetitive, can be employed
in performing an assay method of this invention.

       In one embodiment, the invention contemplates a
direct binding assay using a ligand-binding
heterodimeric receptor of this invention as a binding
35   reagent to detect the presence of a preselected ligand

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with which the receptor binds. The method comprises
the steps of a) admixing a sample suspected to contain
a preselected antigen with a ligand-binding
heterodimeric receptor of this invention that binds to
5   the preselected ligand under binding conditions
sufficient for the ligand-binding heterodimeric
receptor to bind the ligand and form a ligand-receptor
complex; and b) detecting the presence of the ligand-
receptor complex or the ligand-binding heterodimeric
10   receptor in the complex.

Binding conditions are those that maintain the
ligand-binding activity of the receptor. Those
conditions include a temperature range of about 4 to
50 degrees Centigrade, a pH value range of about 5 to
15   9 and an ionic strength varying from about that of
distilled water to that of about one molar sodium
chloride.

The detecting step can be directed, as is well
known in the immunological arts, to either the complex
20   or the binding reagent (the receptor component of the
complex). Thus, a secondary binding reagent such as
an antibody specific for the receptor may be utilized.

Alternatively, the complex may be detectable by
virtue of having used a labeled receptor molecule,
25   thereby making the complex labeled. Detection in this
case comprises detecting the label present in the
complex.

In a preferred embodiment, the ligand-binding
heterodimeric receptor is provided as an attachment on
30   a filamentous phage particle, i.e., on the surface of
the phage. An exemplary assay using a filamentous
phage of this invention is described in an ELISA
format in the Examples.

In another embodiment, a filamentous phage
35   particle is detectably labeled, such as by a

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radioisotope incorporated in a protein or nucleic acid
of the phage as described herein. In this embodiment,
detection comprises detecting the label in the
complex, and thereby detecting the presence of the
ligand in the complex.

    A further diagnostic method utilizes the
multivalency of a filamentous phage particle to cross-
link ligand, thereby forming an aggregation of
multiple ligands and phage particles, producing a
precipitable aggregate. This embodiment is comparable
to the well known methods of immune precipitation.
This embodiment comprises the steps of admixing a
sample with a plurality of phage particle of this
invention to form a binding admixture under binding
conditions, followed by a separation step to isolate
the formed binding complexes. Typically, isolation is
accomplished by centrifugation or filtration to remove
the aggregate from the admixture. The presence of
binding complexes indicates the presence of the
preselected ligand to be detected.

Examples

    The following examples are intended to
illustrate, but not limit, the scope of the invention.

1.   Construction of a Dicistronic Expression Vector
     for Producing a Heterodimeric Receptor on Phage
     Particles

     To obtain a vector system for generating a large
number of Fab antibody fragments that can be screened
directly, expression libraries in bacteriophage Lambda
have previously been constructed as described in Huse
et al., Science, 246:1275-1281 (1989). These systems
did not contain design features that provide for the
expressed Fab to be targeted to the surface of a

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filamentous phage particle.

   The main criterion used in choosing a vector
system was the necessity of generating the largest
number of Fab fragments which could be screened
5   directly. Bacteriophage Lambda was selected as the
starting point to develop an expression vector for
three reasons. First, in vitro packaging of phage DNA
was the most efficient method of reintroducing DNA
into host cells. Second, it was possible to detect
10   protein expression at the level of single phage
plaques. Finally, the screening of phage libraries
typically involved less difficulty with nonspecific
binding. The alternative, plasmid cloning vectors,
are only advantageous in the analysis of clones after
15   they have been identified. This advantage was not
lost in the present system because of the use of a
dicistronic expression vector such as pCombVIII,
thereby permitting a plasmid containing the heavy
chain, light chain, or Fab expressing inserts to be
20   excised.

         a.   Construction of Dicistronic Expression
               Vector pCOMB
               (i)   Preparation of Lambda Zap ^{TM}II
               Lambda Zap^{TM} II is a derivative of the
25   original Lambda Zap (ATCC # 40,298) that maintains all
of the characteristics of the original Lambda Zap
including 6 unique cloning sites, fusion protein
expression, and the ability to rapidly excise the
insert in the form of a phagemid (Bluescript SK-), but
30   lacks the SAM 100 mutation, allowing growth on many
Non-Sup F strains, including XL1-Blue. The Lambda
Zap^{TM} II was constructed as described in Short et al.,
Nuc. Acids Res., 16:7583-7600, 1988, by replacing the
Lambda S gene contained in a 4254 base pair (bp) DNA
35   fragment produced by digesting Lambda Zap with the

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restriction enzyme Nco I. This 4254 bp DNA fragment
was replaced with the 4254 bp DNA fragment containing
the Lambda S gene isolated from Lambda gt10 (ATCC #
40,179) after digesting the vector with the
5   restriction enzyme Nco I. The 4254 bp DNA fragment
isolated from lambda gt10 was ligated into the
original Lambda Zap vector using T4 DNA ligase and
standard protocols such as those described in <u>Current
Protocols in Molecular Biology</u>, Ausubel et al., eds.,
10   John Wiley and Sons, NY, 1987, to form Lambda Zap™
II.

(ii)   <u>Preparation of Lambda Hc2</u>

To express a plurality of V_H-coding DNA
homologs in an <u>E. coli</u> host cell, a vector designated
15   Lambda Hc2 was constructed. The vector provided the
following: the capacity to place the V_H-coding DNA
homologs in the proper reading frame; a ribosome
binding site as described by Shine et al., <u>Nature</u>,
254:34, 1975; a leader sequence directing the
20   expressed protein to the periplasmic space designated
the pelB secretion signal; a polynucleotide sequence
that coded for a known epitope (epitope tag); and also
a polynucleotide that coded for a spacer protein
between the V_H-coding DNA homolog and the
25   polynucleotide coding for the epitope tag. Lambda Hc2
has been previously described by Huse et al., <u>Science</u>,
246:1275-1281 (1989).

To prepare Lambda Hc2, a synthetic DNA sequence
containing all of the above features was constructed
30   by designing single stranded polynucleotide segments
of 20-40 bases that would hybridize to each other and
form the double stranded synthetic DNA sequence shown
in Figure 3. The individual single-stranded
polynucleotide segments are shown in Table 3.
35   Polynucleotides N2, N3, N9-4, N11, N10-5, N6, N7

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and N8 (Table 3) were kinased by adding 1 μl of each
polynucleotide 0.1 micrograms/microliter (μg/μl) and
20 units of T_4 polynucleotide kinase to a solution
containing 70 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM
5   dithiothreitol (DTT), 10 mM beta-mercaptoethanol, 500
micrograms per milliliter (μg/ml) bovine serum albumin
(BSA). The solution was maintained at 37 degrees
Centigrade (37°C) for 30 minutes and the reaction
stopped by maintaining the solution at 65°C for 10
10   minutes. The two end polynucleotides, 20 ng of
polynucleotides N1 and polynucleotides N12, were added
to the above kinasing reaction solution together with
1/10 volume of a solution containing 20.0 mM Tris-HCl,
pH 7.4, 2.0 mM $MgCl_2$ and 50.0 mM NaCl. This solution
15   was heated to 70°C for 5 minutes and allowed to cool
to room temperature, approximately 25°C, over 1.5
hours in a 500 ml beaker of water. During this time
period all 10 polynucleotides annealed to form the
double stranded synthetic DNA insert shown in Figure
20   3. The individual polynucleotides were covalently
linked to each other to stabilize the synthetic DNA
insert by adding 40 μl of the above reaction to a
solution containing 50 mM Tris-HCl, pH 7.5, 7 mM
$MgCl_2$, 1 mM DTT, 1 mM adenosine triphosphate (ATP) and
25   10 units of T4 DNA ligase. This solution was
maintained at 37°C for 30 minutes and then the T4 DNA
ligase was inactivated by maintaining the solution at
65°C for 10 minutes. The end polynucleotides were
kinased by mixing 52 μl of the above reaction, 4 μl of
30   a solution containing 10 mM ATP and 5 units of T4
polynucleotide kinase. This solution was maintained
at 37°C for 30 minutes and then the T4 polynucleotide
kinase was inactivated by maintaining the solution at
65°C for 10 minutes.
35

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Table 3

SEQ.

ID. NO.

(22)	N1)	5' GGCCGCAAATTCTATTTCAAGGAGACAGTCAT 3'
(23)	N2)	5' AATGAAATACCTATTGCCTACGGCAGCCGCTGGATT 3'
(24)	N3)	5' GTTATTACTCGCTGCCCAACCAGCCATGGCCC 3'
(25)	N6)	5' CAGTTTCACCTGGGCCATGGCTGGTTGGG 3'
(26)	N7)	5' CAGCGAGTAATAACAATCCAGCGGCTGCCGTAGGCAATAG 3'
(27)	N8)	5' GTATTTCATTATGACTGTCTCCTTGAAATAGAATTTGC 3'
(28)	N9-4)	5' AGGTGAAACTGCTCGAGATTTCTAGACTAGTTACCCGTAC 3'
(29)	N10-5)	5' CGGAACGTCGTACGGGTAACTAGTCTAGAAATCTCGAG 3'
(30)	N11)	5' GACGTTCCGGACTACGGTTCTTAATAGAATTCG 3'
(31)	N12)	5' TCGACGAATTCTATTAAGAACCGTAGTC 3'

The completed synthetic DNA insert was ligated
directly into the Lambda Zap™ II vector described in
Example 1a(i) that had been previously digested with
the restriction enzymes, Not I and Xho I. The
ligation mixture was packaged according to the
manufacture's instructions using Gigapack II Gold
packing extract available from Stratagene, La Jolla,
California. The packaged ligation mixture was plated
on XL1-Blue cells (Stratagene). Individual lambda
plaques were cored and the inserts excised according
to the in vivo excision protocol for Lambda Zap™ II
provided by the manufacturer (Stratagene). This in
vivo excision protocol moved the cloned insert from
the Lambda Hc2 vector into a phagemid vector to allow
easy for manipulation and sequencing. The accuracy of
the above cloning steps was confirmed by sequencing
the insert using the Sanger dideoxy method described
in by Sanger et al., Proc. Natl. Acad. Sci. USA,
74:5463-5467, (1977) and using the manufacture's
instructions in the AMV Reverse Transcriptase ^{35}S-ATP

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sequencing kit (Stratagene). The sequence of the
resulting double-stranded synthetic DNA insert in the
V_H expression vector (Lambda Hc2) is shown in Figure
3. The sequence of each strand (top and bottom) of
5   Lambda Hc2 is listed in the sequence listing as SEQ ID
NO 1 and SEQ ID NO 2, respectively. The resultant
Lambda Hc2 expression vector is shown in Figure 4.

(iii) Preparation of Lambda Lc2

To express a plurality of V_L-coding
10   DNA homologs in an E. coli host cell, a vector
designated Lambda Lc2 was constructed having the
capacity to place the V_L-coding DNA homologs in the
proper reading frame, provided a ribosome binding site
as described by Shine et al., Nature, 254:34 (1975),
15   provided the pelB gene leader sequence secretion
signal that has been previously used to successfully
secrete Fab fragments in E. coli by Lei et al., J.
Bac., 169:4379 (1987) and Better et al., Science,
240:1041 (1988), and also provided a polynucleotide
20   containing a restriction endonuclease site for
cloning. Lambda Lc2 has been previously described by
Huse et al., Science, 246:1275-1281 (1989).

A synthetic DNA sequence containing all of the
above features was constructed by designing single
25   stranded polynucleotide segments of 20-60 bases that
would hybridize to each other and form the double
stranded synthetic DNA sequence shown in Figure 5.
The sequence of each individual single-stranded
polynucleotide segment (01-08) within the double
30   stranded synthetic DNA sequence is shown in Table 4.

Polynucleotides 02, 03, 04, 05, 06 and 07 (Table
4) were kinased by adding 1 μl (0.1 ug/μl) of each
polynucleotide and 20 units of T_4 polynucleotide
kinase to a solution containing 70 mM Tris-HCl, pH
35   7.6, 10 mM MgCl, 5 mM DTT, 10 mM beta-mercaptoethanol,

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500 mg/ml of BSA. The solution was maintained at 37°C
for 30 minutes and the reaction stopped by maintaining
the solution at 65°C for 10 minutes. The 20 ng each
of the two end polynucleotides, 01 and 08, were added
5   to the above kinasing reaction solution together with
1/10 volume of a solution containing 20.0 mM Tris-HCl,
pH 7.4, 2.0 mM MgCl and 15.0 mM sodium chloride
(NaCl). This solution was heated to 70°C for 5
minutes and allowed to cool to room temperature,
10   approximately 25°C, over 1.5 hours in a 500 ml beaker
of water. During this time period all 8
polynucleotides annealed to form the double stranded
synthetic DNA insert shown in Figure 5. The
individual polynucleotides were covalently linked to
15   each other to stabilize the synthetic DNA insert by
adding 40 μl of the above reaction to a solution
containing 50 ml Tris-HCl, pH 7.5, 7 ml MgCl, 1 mm
DTT, 1 mm ATP and 10 units of T4 DNA ligase. This
solution was maintained at 37°C for 30 minutes and
20   then the T4 DNA ligase was inactivated by maintaining
the solution at 65°C for 10 minutes. The end
polynucleotides were kinased by mixing 52 μl of the
above reaction, 4 μl of a solution containing 10 mM
ATP and 5 units of T4 polynucleotide kinase. This
25   solution was maintained at 37°C for 30 minutes and
then the T4 polynucleotide kinase was inactivated by
maintaining the solution at 65°C for 10 minutes.

                        TABLE 4

30   SEQ.
       ID. NO.
       (32) 01)   5' TGAATTCTAAACTAGTCGCCAAGGAGACAGTCAT 3'
       (33) 02)   5' AATGAAATACCTATTGCCTACGGCAGCCGCTGGATT 3'
       (34) 03)   5' GTTATTACTCGCTGCCCAACCAGCCATGGCC 3'
35   (35) 04)   5' GAGCTCGTCAGTTCTAGAGTTAAGCGGCCG 3'

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(36) 05)   5' GTATTTCATTATGACTGTCTCCTTGGCGACTAGTTTAGAA-
                TTCAAGCT 3'

(37) 06)   5' CAGCGAGTAATAACAATCCAGCGGCTGCCGTAGGCAATAG
           3'

5   (38) 07)   5' TGACGAGCTCGGCCATGGCTGGTTGGG 3'

(39) 08)   5' TCGACGGCCGCTTAACTCTAGAAC 3'

        The completed synthetic DNA insert was ligated
10 directly into the Lambda Zap™ II vector described in
    Example 1(a)(i) that had been previously digested with
    the restriction enzymes Sac I and Xho I. The ligation
    mixture was packaged according to the manufacture's
    instructions using Gigapack II Gold packing extract
15 (Stratagene). The packaged ligation mixture was
    plated on XL1-Blue cells (Stratagene). Individual
    lambda plaques were cored and the inserts excised
    according to the in vivo excision protocol for Lambda
    Zap™ II provided by the manufacturer (Stratagene).
20 This in vivo excision protocol moved the cloned insert
    from the Lambda Lc2 vector into a plasmid phagemid
    vector allow for easy manipulation and sequencing.
    The accuracy of the above cloning steps was confirmed
    by sequencing the insert using the manufacture's
25 instructions in the AMV Reverse Transcriptase ^{35}S-dATP
    sequencing kit (Stratagene). The sequence of the
    resulting Lc2 expression vector (Lambda Lc2) is shown
    in Figure 5. Each strand is separately listed in the
    Sequence Listing as SEQ ID NO 3 and SEQ ID NO 4. The
30 resultant Lc2 vector is schematically diagrammed in
    Figure 6.
        A preferred vector for use in this invention,
    designated Lambda Lc3, is a derivative of Lambda Lc2
    prepared above. Lambda Lc2 contains a Spe I
35 restriction site (ACTAGT) located 3' to the EcoR I

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restriction site and 5' to the Shine-Dalgarno ribosome
binding site as shown in the sequence in Figure 5 and
in SEQ ID NO 3. A Spe I restriction site is also
present in Lambda Hc2 as shown in Figures 3 and 4 and
in SEQ ID NO 1. A combinatorial vector, designated
pComb, was constructed by combining portions of Lambda
Hc2 and Lc2 together as described in Example 1a(iv)
below. The resultant combinatorial pComb vector
contained two Spe I restriction sites, one provided by
Lambda Hc2 and one provided by Lambda Lc2, with an
EcoR I site in between. Despite the presence of two
Spe I restriction sites, DNA homologs having Spe I and
EcoR I cohesive termini were successfully
directionally ligated into a pComb expression vector
previously digested with Spe I and EcoR I as described
in Example 1b below. The proximity of the EcoR I
restriction site to the 3' Spe I site, provided by the
Lc2 vector, inhibited the complete digestion of the 3'
Spe I site. Thus, digesting pComb with Spe I and EcoR
I did not result in removal of the EcoR I site between
the two Spe I sites.

        The presence of a second Spe I restriction site
may be undesirable for ligations into a pComb vector
digested only with Spe I as the region between the two
sites would be eliminated. Therefore, a derivative of
Lambda Lc2 lacking the second or 3' Spe I site,
designated Lambda Lc3, is produced by first digesting
Lambda Lc2 with Spe I to form a linearized vector.
The ends are filled in to form blunt ends which are
ligated together to result in Lambda Lc3 lacking a Spe
I site. Lambda Lc3 is a preferred vector for use in
constructing a combinatorial vector as described
below.

                (iv) Preparation of pComb
                      Phagemids were excised from the

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expression vectors Lambda Hc2 or Lambda Lc2 using an
in vivo excision protocol described above. Double
stranded DNA was prepared from the phagemid-containing
cells according to the methods described by Holmes et
5   al., <u>Anal. Biochem.</u>, 114:193 (1981). The phagemids
resulting from *in vivo* excision contained the same
nucleotide sequences for antibody fragment cloning and
expression as did the parent vectors, and are
designated phagemid Hc2 and Lc2, corresponding to
10   Lambda Hc2 and Lc2, respectively.
        For the construction of combinatorial phagemid
vector pComb, produced by combining portions of
phagemid Hc2 and phagemid Lc2, phagemid Hc2 was first
digested with Sac I to remove the restriction site
15   located 5' to the LacZ promoter. The linearized
phagemid was then blunt ended with T4 polymerase and
ligated to result in a Hc2 phagemid lacking a Sac I
site. The modified Hc2 phagemid and the Lc2 phagemid
were then separately restriction digested with Sca I
20   and EcoR I to result in a Hc2 fragment having from 5'
to 3' Sca I, Not I Xho I, Spe I and EcoR I restriction
sites and a Lc2 fragment having from 5' to 3' EcoR I,
Sac I, Xba I and Sac I restriction sites. The
linearized phagemids were then ligated together at
25   their respective cohesive ends to form pComb, a
circularized phagemid having a linear arrangement of
restriction sites of Not I, Xho I, Spe I, EcoR I, Sac
I, Xba I, Not I, Apa I and Sca I. The ligated
phagemid vector was then inserted into an appropriate
30   bacterial host and transformants were selected on the
antibiotic ampicillin.
        Selected ampicillin resistant transformants were
screened for the presence of two Not I sites. The
resulting ampicillin resistant combinatorial phagemid
35   vector was designated pComb, the schematic

organization of which is shown in Figure 7. The
resultant combinatorial vector, pComb, consisted of a
DNA molecule having two cassettes to express two
fusion proteins and having nucleotide residue
5   sequences for the following operatively linked
elements listed in a 5' to 3' direction: a first
cassette consisting of an inducible LacZ promoter
upstream from the LacZ gene; a Not I restriction site;
a ribosome binding site; a pelB leader; a spacer; a
10   cloning region bordered by a 5' Xho and 3' Spe I
restriction site; a decapeptide tag followed by
expression control stop sequences; an EcoR I
restriction site located 5' to a second cassette
consisting of an expression control ribosome binding
15   site; a pelB leader; a spacer region; a cloning region
bordered by a 5' Sac I and a 3' Xba I restriction site
followed by expression control stop sequences and a
second Not I restriction site.

A preferred combinatorial vector for use in this
20   invention, designated pComb2, is constructed by
combining portions of phagemid Hc2 and phagemid Lc3 as
described above for preparing pComb. The resultant
combinatorial vector, pComb2, consists of a DNA
molecule having two cassettes identical to pComb to
25   express two fusion proteins identically to pComb
except that a second Spe I restriction site in the
second cassette is eliminated.

      b.   Construction of Vectors pCombVIII and
            pCombIII for Expressing Fusion Proteins
30   Having a Bacteriophage Coat Protein Membrane
            Anchor

Because of the multiple endonuclease
restriction cloning sites, the pComb phagemid
expression vector prepared above is a useful cloning
35   vehicle for modification for the preparation of an

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expression vector of this invention. To that end,
pComb is digested with EcoR I and Spe I followed by
phosphatase treatment to produce linearized pComb.

(i)   Preparation of pCombVIII

A PCR product produced in Example 2g
and having a nucleotide sequence that defines a
filamentous bacteriophage coat protein VIII (cpVIII)
membrane anchor domain and cohesive Spe I and EcoR I
termini was admixed with the linearized pComb to form
a ligation admixture. The cpVIII-membrane anchor-
encoding PCR fragment was directionally ligated into
the pComb phagemid expression vector at corresponding
cohesive termini, that resulted in forming pCombVIII
(also designated pComb8). pCombVIII contains a
cassette defined by the nucleotide sequence shown in
SEQ ID NO 116 from nucleotide base 1 to base 208, and
contains a pelB secretion signal operatively linked to
the cpVIII membrane anchor.

A preferred phagemid expression vector for use in
this invention, designated either pComb2-VIII or
pComb2-8, was prepared as described above by
directionally ligating the cpVIII membrane anchor-
encoding PCR fragment into a pComb2 phagemid
expression vector via Spe I and EcoR I cohesive
termini. The pComb2-8 had only one Spe I restriction
site.

(ii)   Preparation of pCombIII

A separate phagemid expression vector
was constructed using sequences encoding bacteriophage
cpIII membrane anchor domain. A PCR product defining
the cpIII membrane anchor containing a LacZ promotor
region sequence 3' to the membrane anchor for
expression of the light chain and Spe I and EcoR I
cohesive termini was prepared as described for cpVIII,
the details of which are described in Example 2g. The

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cpIII-derived PCR product was then ligated into
linearized pComb2 vector having only one Spe I site to
form the vector pComb2-3 (also designated pComb2-III).

A more preferred phagemid expression vector for
use in this invention having additional restriction
enzyme cloning sites, designated pComb-III' or pComb2-
3', was prepared as described above for pComb2-3 with
the addition of a 51 base pair fragment from
pBluescript as described by Short et al., Nuc. Acids
Res., 16:7583-7600 (1988) and commercially available
from Stratagene. To prepare pComb2-3', pComb2-3 was
first digested with Xho I and Spe I restriction
enzymes to form a linearized pComb2-3. The vector
pBluescript was digested with the same enzymes
releasing a 51 base pair fragment containing the
restriction enzyme sites Sal I, Acc I, Hinc II, Cla I,
Hind III, EcoR V, Pst I, Sma I and BamH I. The 51
base pair fragment was ligated into the linearized
pComb2-3 vector via the cohesive Xho I and Spe I
termini to form pComb2-3'.

c.   Construction of pCBAK Vectors Having a
      Chloramphenicol Resistance Marker

In order to utilize a different selectable
marker gene, such as chloramphenicol acetyl
transferase (CAT), for the selection of bacteria
transformed with a vector of this invention,
expression vectors based on pComb were developed
having a gene encoding CAT and are designated pCBAK
vectors. The pCBAK vectors are prepared by combining
portions of pCB and pComb.

(i) Preparation of pCB

pBlueScript phagemid vectors, pBC SK(-)
and pBS SK(-), (Stratagene), were modified and
combined to generate a third vector designated pCB as
described below.

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pBC SK(-), which contains a chloramphenicol
resistance selectable marker gene, was digested with
Bst BI and blunt ended with T4 polymerase. A second
digestion with Pvu I allowed for the removal of a 1
5   kilobase (kb) fragment leaving a 2.4 kb linearized
vector which retained the CAT selectable resistance
marker gene, an inducible LacZ promoter upstream from
the LacZ gene and a ColE1 origin region. The 2.4 kb
fragment was recovered. The pBS SK(-) vector was
10   digested with Aat II and blunt ended with T4
polymerase. A second digestion with Pvu I allowed for
the isolation of an 800 base pair (bp) fragment
containing the f1 origin of replication. Ligation of
the pBS derived 800 bp f1 fragment with the 2.4 kb pBC
15   fragment created a pCB precursor vector containing a
Sac I site, an f1 origin of replication, a CAT
selectable resistance marker gene, ColE1 origin, a
multiple cloning site (MCS) flanked by T_3 and T_7
promoters, and an inducible LacZ promoter upstream
20   from LacZ gene.

The pCB precursor vector was then digested with
Sac I and blunt-ended with T4 polymerase. The T4
polymerase-treated pCB vector was then religated to
form pCB vector and is lacking a Sac I site.

25   (ii)   Preparation of pCBAKO

The pCB vector containing the CAT
selectable resistance marker gene was digested with
Sac II and Apa I and treated with phosphatase to
prevent religation and to form linearized pCB vector.
30   The pComb vector prepared in Example 1(a)(iv) was
restriction digested with Sac II and Apa I to release
a fragment containing nucleotide residue sequences
starting 5' to the LacZ promoter and extending past
the 3' end of the second Not I site. The Sac II and
35   Apa I pComb DNA fragment was then directionally

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ligated into the similarly digested pCB vector to form
phagemid expression vector pCBAK0. Preferred pCBAK
expression vectors are constructed with pComb2. The
resultant pCBAK expression vector contained only one

5   Spe I restriction site.

        (iii) Preparation of pCBAK8
                To prepare a pCBAK-based phagemid
expression vector which encodes a bacteriophage coat
protein membrane anchor domain in the expressed fusion

10   protein, pCB phagemid cloning vector prepared in
Example 1c(ii) was linearized by digestion with Sac II
and Apa I. The pCombVIII phagemid expression vector,
prepared in Example 1b(i), was restriction digested
with Sac II and Apa I to form a fragment containing a

15   nucleotide residue sequence starting 5' to the LacZ
promoter and extending past the 3' end of the second
Not 1 site. The fragment was directionally ligated
into the linearized pCB cloning vector to form
phagemid expression vector pCBAK8.

20   (iv)   Preparation of pCBAK3
                The phagemid expression vector,
pCBAK3, for the expression of fusion protein having
cpIII membrane anchor domains, was similarly
constructed by directionally ligating the Sac II and

25   Apa I restriction digested fragment from pCombIII with
Sac II and Apa I linearized pCB cloning vector.

2.   Construction of Dicistronic Expression Vectors
     for Expressing Anti-NPN Heterodimer on Phage
30   Surfaces
        In practicing this invention, the heavy (Fd
consisting of V_H and C_H1) and light (kappa) chains (V_L,
C_L) of antibodies are first targeted to the periplasm
of E. coli for the assembly of heterodimeric Fab
35   molecules. In order to obtain expression of antibody

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Fab libraries on a phage surface, the nucleotide
residue sequences encoding either the Fd or light
chains must be operatively linked to the nucleotide
residue sequence encoding a filamentous bacteriophage
5   coat protein membrane anchor. Two preferred coat
proteins for use in this invention in providing a
membrane anchor are VIII and III (cpVIII and cpIII,
respectively). In the Examples described herein,
methods for operatively linking a nucleotide residue
10   sequence encoding a Fd chain to either cpVIII or cpIII
membrane anchors in a fusion protein of this invention
are described.

In a phagemid vector, a first and second cistron
consisting of translatable DNA sequences are
15   operatively linked to form a dicistronic DNA molecule.
Each cistron in the dicistronic DNA molecule is linked
to DNA expression control sequences for the coordinate
expression of a fusion protein, Fd-cpVIII or Fd-cpIII,
and a kappa light chain.

20   The first cistron encodes a periplasmic secretion
signal (pelB leader) operatively linked to the fusion
protein, either Fd-cpVIII or Fd-cpIII. The second
cistron encodes a second pelB leader operatively
linked to a kappa light chain. The presence of the
25   pelB leader facilitates the coordinated but separate
secretion of both the fusion protein and light chain
from the bacterial cytoplasm into the periplasmic
space.

The process described above is schematically
30   diagrammed in Figure 8. Briefly, the phagemid
expression vector carries a chloramphenicol acetyl
transferase (CAT) selectable resistance marker gene in
addition to the Fd-cpVIII fusion and the kappa chain.
The f1 phage origin of replication facilitates the
35   generation of single stranded phagemid. The isopropyl

thiogalactopyranoside (IPTG) induced expression of a
dicistronic message encoding the Fd-cpVIII fusion (V_H,
C_{H1}, cpVIII) and the light chain (V_L, C_L) leads to the
formation of heavy and light chains. Each chain is
delivered to the periplasmic space by the pelB leader
sequence, which is subsequently cleaved. The heavy
chain is anchored in the membrane by the cpVIII
membrane anchor domain while the light chain is
secreted into the periplasm. The heavy chain in the
presence of light chain assembles to form Fab
molecules. This same result can be achieved if, in
the alternative, the light chain is anchored in the
membrane via a light chain fusion protein having a
membrane anchor and heavy chain is secreted via a pelB
leader into the periplasm.

   With subsequent infection of _E. coli_ with a
helper phage, as the assembly of the filamentous
bacteriophage progresses, the coat protein VIII is
incorporated along the entire length of the
filamentous phage particles as shown in Figures 8 and
9. If cpIII is used, the accumulation occurs on the
tail of the bacteriophage. The advantage of the
utilization of membrane anchors from cpVIII over cpIII
is two fold. Firstly, a multiplicity of binding
sites, consisting of approximately 2700 cpVIII
monomers assembled in a tubular array, exist along the
particle surface. Secondly, the construct does not
interfere with phage infectivity.

   a.   Polynucleotide Selection

   The nucleotide sequences encoding the
immunoglobulin protein CDR's are highly variable.
However, there are several regions of conserved
sequences that flank the V region domains of either
the light or heavy chain, for instance, and that
contain substantially conserved nucleotide sequences,

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i.e., sequences that will hybridize to the same primer
sequence. Therefore, polynucleotide synthesis
(amplification) primers that hybridize to the
conserved sequences and incorporate restriction sites
into the DNA homolog produced that are suitable for
operatively linking the synthesized DNA fragments to a
vector were constructed. More specifically, the
primers are designed so that the resulting DNA
homologs produced can be inserted into an expression
vector of this invention in reading frame with the
upstream translatable DNA sequence at the region of
the vector containing the directional ligation means.

(i) V_H Primers

For amplification of the V_H domains,
primers are designed to introduce cohesive termini
compatible with directional ligation into the unique
Xho I and Spe I sites of the phagemid Hc2 expression
vector. For example, the 3' primer (primer 12A in
Table 5), was designed to be complementary to the mRNA
in the J_H region. In all cases, the 5' primers
(primers 1-10, Table 5) were chosen to be
complementary to the first strand cDNA in the
conserved N-terminus region (antisense strand).
Initially amplification was performed with a mixture
of 32 primers (primer 1, Table 5) that were degenerate
at five positions. Hybridoma mRNA could be amplified
with mixed primers, but initial attempts to amplify
mRNA from spleen yielded variable results. Therefore,
several alternatives to amplification using the mixed
5' primers were compared.

The first alternative was to construct multiple
unique primers, eight of which are shown in Table 5,
corresponding to individual members of the mixed
primer pool. The individual primers 2-9 of Table 5
were constructed by incorporating either of the two

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possible nucleotides at three of the five degenerate positions.

The second alternative was to construct a primer containing inosine (primer 10, Table 5) at four of the variable positions based on the published work of Takahashi, et al., _Proc. Natl. Acad. Sci. (USA)_, 82:1931-1935, (1985) and Ohtsuka et al., _J. Biol. Chem._, 260: 2605-2608, (1985). This primer has the advantage that it is not degenerate and, at the same time minimizes the negative effects of mismatches at the unconserved positions as discussed by Martin et al., _Nuc. Acids Res._, 13:8927 (1985). However, it was not known if the presence of inosine nucleotides would result in incorporation of unwanted sequences in the cloned V_H regions. Therefore, inosine was not included at the one position that remains in the amplified fragments after the cleavage of the restriction sites. As a result, inosine was not in the cloned insert.

Additional V_H amplification primers including the unique 3' primer were designed to be complementary to a portion of the first constant region domain of the gamma 1 heavy chain mRNA (primers 16 and 17, Table 5). These primers will produce DNA homologs containing polynucleotides coding for amino acids from the V_H and the first constant region domains of the heavy chain. These DNA homologs can therefore be used to produce Fab fragments rather than F_V.

Additional unique 3' primers designed to hybridize to similar regions of another class of immunoglobulin heavy chain such as IgM, IgE and IgA are contemplated. Other 3' primers that hybridize to a specific region of a specific class of CH_1 constant region and are adapted for transferring the V_H domains amplified using this primer to an expression vector

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capable of expressing those V_H domains with a
different class of heavy or light chain constant
region are also contemplated.

As a control for amplification from spleen or
hybridoma mRNA, a set of primers hybridizing to a
highly conserved region within the constant region
IgG, heavy chain gene were constructed. The 5' primer
(primer 11, Table 5) is complementary to the cDNA in
the C_H2 region whereas the 3' primer (primer 13, Table
5) is complementary to the mRNA in the C_H3 region. It
is believed that no mismatches were present between
these primers and their templates.

The primers used for amplification of heavy chain
Fd fragments for construction of Fabs are shown at
least in Table 5. Amplification was performed in
eight separate reactions, each containing one of the
5' primers (primers 2-9) and one of the 3' primers
(primer 16). The remaining 5' primers that have been
used for amplification in a single reaction are either
a degenerate primer (primer 1) or a primer that
incorporates inosine at four degenerate positions
(primer 10, Table 5, and primers 17 and 18, Table 6).
The remaining 3' primer (primer 14, Table 6) has been
used to construct F_v fragments. Many of the 5'
primers incorporate a Xho I site, and the 3' primers
incorporate a Spe I restriction site for insertion of
the V_H DNA homolog into the phagemid Hc2 expression
vector (Figure 4).

V_H amplification primers designed to amplify
human heavy chain variable regions are shown in Table
6. One of the 5' heavy chain primer contains inosine
residues at degenerate nucleotide positions allowing a
single primer to hybridize to a large number of
variable region sequences. Primers designed to
hybridize to the constant region sequences of various

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IgG mRNAs are also shown in Table 6.

        (ii) V_L Primers

        The nucleotide sequences encoding the V_L CDRs are highly variable. However, there are several regions of conserved sequences that flank the V_L CDR domains including the J_L, V_L framework regions and V_L leader/promotor. Therefore, amplification primers were constructed that hybridized to the conserved sequences and incorporate restriction sites that allow cloning the amplified fragments into the phagemid Lc2 vector cut with Sac I and Xba I.

        For amplification of the V_L CDR domains, the 5' primers (primers 1-8 in Table 6) were designed to be complementary to the first strand cDNA in the conserved N-terminus region. These primers also introduced a Sac I restriction endonuclease site to allow the V_L DNA homolog to be cloned into the phagemid Lc2 expression vector. The 3' V_L amplification primer (primer 9 in Table 6) was designed to be complementary to the mRNA in the J_L regions and to introduce the Xba I restriction endonuclease site required to insert the V_L DNA homolog into the phagemid Lc2 expression vector (Figure 6).

        Additional 3' V_L amplification primers were designed to hybridize to the constant region of either kappa or lambda mRNA (primers 10 and 11 in Table 6). These primers allow a DNA homolog to be produced containing polynucleotide sequences coding for constant region amino acids of either kappa or lambda chain. These primers make it possible to produce an Fab fragment rather than an F_v.

        The primers used for amplification of kappa light chain sequences for construction of Fabs are shown at least in Table 6. Amplification with these primers

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was performed in 5 separate reactions, each containing one of the 5' primers (primers 3-6, and 12) and one of the 3' primers (primer 13). The remaining 3' primer (primer 9) has been used to construct F_v fragments.

5   The 5' primers contain a Sac I restriction site and the 3' primers contain a Xba I restriction site.

V_L amplification primers designed to amplify human light chain variable regions of both the lambda and kappa isotypes are also shown in Table 6.

10   All primers and synthetic polynucleotides described herein, including those shown in Tables 3-7 were either purchased from Research Genetics in Huntsville, Alabama or synthesized on an Applied Biosystems DNA synthesizer, model 381A, using the

15   manufacturer's instruction.

TABLE 5

(1)	5'AGGT(C/G)(C/A)A(G/A)CT(G/T)CTCGAGTC(T/A)GG 3'	degenerate 5' primer for the amplification of mouse and human heavy chain variable regions (V_H)
(2)	5'AGGTCCAGCTGCTCGAGTCTGG 3'	Unique 5' primer for the amplification of mouse and human V_H
(3)	5'AGGTCCAGCTGCTCGAGTCAGG 3'	=
(4)	5'AGGTCCAGCTTCTCGAGTCTGG 3'	=
(5)	5'AGGTCCAGCTTCTCGAGTCAGG 3'	=
(6)	5'AGGTCCAACTGCTCGAGTCTGG 3'	=
(7)	5'AGGTCCAACTGCTCGAGTCAGG 3'	=
(8)	5'AGGTCCAACTTCTCGAGTCTGG 3'	=
(9)	5'AGGTCCAACTTCTCGAGTCAGG 3'	=
(10)	5'AGGTIIAICTTCTCGAGTC(T/A)GG 3'	5' degenerate primer containing inosine at 4 degenerate positions for amplification of mouse V_H
(11)	5'GCCCAAGGATGTGCTCACC 3'	5' primer for amplification in the C_H2 region of mouse IgG1
(12)	5'CTATTAGAATTCAACGGTAACAGTGGTGCCTTGGCCCCA 3'	3' primer for amplification of V_H and introducing a 3' Eco RI site
(12A)	5'CTATTAACTAGTAACGGTAACAGTGGTGCCTTGGCCCCA 3'	3' primer for amplification of V_H using 3' Spe I site
(13)	5'CTCAGTATGGTGGTTGTGC 3'	3' primer for amplification in the C_H3 region of mouse IgG1
(14)	5'GCTACTAGTTTTGATTCCACCTTGG 3'	3' primer for amplification of mouse kappa light chain variable regions (V_L)
(15)	5'CAGCCATGGCCGACATTCCAGATG 3'	5' primer for amplification of mouse kappa light chain variable regions

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TABLE 5 - Continued

(16) 5'AATTTTACTAGTCACCTTGGTGTGCTGCTGGC 3'

Unique 3' primer for amplification of V$_H$ including part of the mouse gamma 1 first constant region

(17) 5'TATGCAACTAGTACAACCACAATCCCTGGGCACAATTTT 3'

Unique 3' primer for amplification of Fd including part of mouse IgG1 first constant region and hinge region

(10) 5'AGGCTTACTAGTACAATCCCTGGGCACAAT 3'

3' primer for amplifying mouse Fd including part of the mouse IgG first constant region and part of the hinge region

TABLE 6

(1)	5' CCAGTTCCGAGCTCGTTGTGTGACTCAGGAATCT 3'	Unique 5' primer for the amplification of kappa light chain variable regions
(2)	5' CCAGTTCCGAGCTCGTGTTGACGCAGCCGCCC 3'	"
(3)	5' CCAGTTCCGAGCTCGTGCTCACCCAGTCTCCA 3'	"
(4)	5' CCAGTTCCGAGCTCCAGATGACCCAGTCTCCA 3'	"
(5)	5' CCAGATGTGAGCTCGTGATGACCCAGACTCCA 3'	"
(6)	5' CCAGATGTGAGCTCGTCATGACCCAGTCTCCA 3'	"
(7)	5' CCAGATGTGAGCTCTTGATGACCCAAACTCAA 3'	"
(8)	5' CCAGATGTGAGCTCGTGATAACCCAGGATGAA 3'	"
(9)	5' GCAGCATTCTAGAGTTTCAGCTCCAGCTTGCC 3'	Unique 3' primer for amplification of kappa light chain variable regions
(10)	5' CCGCCGTCTAGAACACTCATTCCTGTTGAAGCT 3'	Unique 3' primer for mouse kappa light chain amplification including the constant region
(11)	5' CCGCCGTCTAGAACATTCTGCAGGAGACAGACT 3'	Unique 3' primer for mouse lambda light chain amplification including the constant region
(12)	5' CCAGTTCCGAGCTCGTGATGACACAGTCTCCA 3'	Unique 5' primer for V_L amplification
(13)	5' GCGCCGTCTAGAATTAACACTCATTCCTGTTGAA 3'	Unique 3' primer for amplification of kappa light chain
(14)	5' CTATTAACTAGTAACGGTAACAGTGGTGCCTTGCCCCCA 3'	Unique 3' primer for amplification of mouse F_V
(15)	5' AGGCTTACTAGTACAATCCCTGGGCACAAT 3'	Unique 3' primer for amplification of mouse IgG Fd
(16)	5' GCCGCTCTAGAACACTCATTCCTGTTGAA 3'	Unique 3' primer for amplification of mouse kappa light chain

TABLE 6 — Continuation Page 1

No.	Sequence	Description
(17)	5' AGGTIIAICTICTCGAGTCTGC 3'	Degenerate 5' primer containing inosine at 4 degenerate positions for amplifying mouse V_H
(18)	5' AGGTIIAICTICTCGAGTCAGC 3'	"
(19)	5' GTGCCAGATGTGAGCTCGTGATGACCCAGTCTCCA 3'	Unique 5' primer for human and mouse kappa V_L amplification
(20)	5' TCCTTCTAGATTACTAACACTCTCCCCTGTTGAA 3'	Unique 3' primer for kappa V_L amplification
(21)	5' GCATTCTAGACTATTATGAACATTCTGTAGGGGC 3'	Unique 3' primer for human, mouse and rabbit-lambda V_L amplification
(22)	5' CTGCACAGGGTCCTGGCCCGAGCTCGTGGTGACTCAG 3'	Unique 5' primer for human lambda V_L amplification
(23)	5' AGITGCAIITGCTCGAGTCTGG 3'	5' degenerate primer for human V_H amplification containing inosine at 3 degenerate positions
(24)	5' GTGGGCATGTGTGAGTTGTGTCACTAGTTGGGGTTTTGAGCTC 3'	Unique 3' primer for human V_H amplification
(25)	5' AGCATCACTAGTACAAGATTTGGGCTC 3'	Unique 3' primer for human IgG1 Fd amplification
(26)	5' AGGTGCAGCTGCTCGAGTCTGG 3'	Unique 5' primers for amplification of human variable regions (V_H)
(27)	5' AGGTGCAGCTGCTCGAGTCGGG 3'	"
(28)	5' AGGTGCAACTGCTCGAGTCTGG 3'	"
(29)	5' AGGTGCAACTGCTCGAGTCGGG 3'	"

TABLE 6 - Continuation Part 2

(30)   5' TCCTTCTTAGATTACTAACACTCTCCCCTGTTGAA 3'   3' primer in human kappa light chain constant region

(31)   5' CTGCACAGGGTCCTGGGCCGAGCTCGTGGTGACTCAG 3'   5' primer for amplification of human lambda light chain variable regions

(32)   5' GCATTCTAGACTATTAACATTCTGTAGGGGC 3'   3' primer in human lambda light chain constant region

(33)   5' ACCCAAGGACACCCCTCATG 3'   Control primer hybridizing to the human CH$_2$ region

(34)   5' CTCAGTATGGTGGTTGTGC 3'   Control primer hybridizing to the human CH$_3$ region

(35)   5' GTCTCCACTAGTCTCCACCAAGGGCCCATCGGTC 3'   5' primer for amplifying human IgG heavy chain variable regions

(36)   5' ATATACTAGTGAGACAGTGACCAGGGTTCCTTGGCCCCA 3'   3' primer for amplifying human heavy chain first constant region

(37)   5' ACGTCTAGATTCCACCTTGGTCCC 3'   3' primer for amplifying human kappa chain variable regions

(38)   5' GCATACTAGTCTATTAACATTCTGTAGGGGC 3'   5' primer for amplifying human kappa light chain constant region

(39)   5' CCGGAATTCTTATCATTTACCCGGAGA 3'   3' primer located in the CH3 region of human IgG1 to amplify the entire heavy chain

(40)   5' TCTGCACTAGTTGGAATGGGCACATGCAG 3'   3' primer for amplifying the Fd region of mouse IgM

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The 19 primers listed in Table 5 have been listed in the Sequence Listing and have been assigned the following SEQ ID NO:

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      (1)  = SEQ ID NO  40
      (2)  = SEQ ID NO  41
 5    (3)  = SEQ ID NO  42
      (4)  = SEQ ID NO  43
      (5)  = SEQ ID NO  44
      (6)  = SEQ ID NO  45
10    (7)  = SEQ ID NO  46
      (8)  = SEQ ID NO  47
      (9)  = SEQ ID NO  48
     (10)  = SEQ ID NO  49
     (11)  = SEQ ID NO  50
15   (12)  = SEQ ID NO  51
     (12A) = SEQ ID NO  52
     (13)  = SEQ ID NO  53
     (14)  = SEQ ID NO  54
     (15)  = SEQ ID NO  55
20   (16)  = SEQ ID NO  56
     (17)  = SEQ ID NO  57
     (18)  = SEQ ID NO  58
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25   The 40 primers listed as "(1)" through "(40)" in Table 6 have also been individually and sequentially listed in the Sequence Listing beginning with SEQ ID NO 59 through SEQ ID NO 98, respectively.

        b.   Preparation of a Repertoire of Genes
30   Encoding Immunoglobulin Variable Domain
              Nitrophenylphosphonamidate (NPN) was selected as the ligand for receptor binding in preparing a heterodimeric receptor according to the methods of the invention.

35   Keyhole limpet hemocyanin (KLH) was conjugated to

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NPN to form a NPN-KLH conjugate used for immunizing a
mouse to produce an anti-NPN immune response and
thereby provide a source of ligand specific
heterodimeric receptor genes.

5   The NPN-KLH conjugate was prepared by admixing
250 µl of a solution containing 2.5 mg of NPN in
dimethylformamide with 750 µl of a solution containing
2 mg of KLH in 0.01 Molar (M) sodium phosphate buffer
(pH 7.2). The two solutions were admixed by slow

10   addition of the NPN solution to the KLH solution while
the KLH solution was being agitated by a rotating
stirring bar. Thereafter the admixture was maintained
at 4°C for 1 hour with the same agitation to allow
conjugation to proceed. The conjugated NPN-KLH was

15   isolated from the nonconjugated NPN and KLH by gel
filtration through Sephadex G-25. The isolated NPN-
KLH conjugate was injected into mice as described
below.

              The NPN-KLH conjugate was prepared for injection

20   into mice by adding 100 µg of the conjugate to 250 µl
of phosphate buffered saline (PBS). An equal volume
of complete Freund's adjuvant was added and emulsified
the entire solution for 5 minutes. A 129 G_{IX+} mouse
was injected with 300 µl of the emulsion. Injections

25   were given subcutaneously at several sites using a 21
gauge needle. A second immunization with NPN-KLH was
given two weeks later. This injection was prepared as
follows: 50 micrograms (µg) of NPN-KLH were diluted
in 250 µl of PBS and an equal volume of alum was

30   admixed to the NPN-KLH solution. The mouse was
injected intraperitoneally with 500 µl of the solution
using a 23 gauge needle. One month later the mice
were given a final injection of 50 µg of the NPN-KLH
conjugate diluted to 200 µl in PBS. This injection

35   was given intravenously in the lateral tail vein using

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a 30 gauge needle. Five days after this final
injection the mice were sacrificed and total cellular
RNA was isolated from their spleens.

Total cellular RNA was prepared from the spleen
of a single mouse immunized with KLH-NPN as described
above using the RNA preparation methods described by
Chomczynski et al., Anal_Biochem., 162:156-159 (1987)
and using the RNA isolation kit (Stratagene) according
to the manufacturer's instructions. Briefly,
immediately after removing the spleen from the
immunized mouse, the tissue was homogenized in 10 ml
of a denaturing solution containing 4.0 M guanine
isothiocyanate, 0.25 M sodium citrate at pH 7.0, and
0.1 M beta-mercaptoethanol using a glass homogenizer.
One ml of sodium acetate at a concentration of 2 M at
pH 4.0 was admixed with the homogenized spleen. One
ml of phenol that had been previously saturated with
H_2O was also admixed to the denaturing solution
containing the homogenized spleen. Two ml of a
chloroform:isoamyl alcohol (24:1 v/v) mixture was
added to this homogenate. The homogenate was mixed
vigorously for ten seconds and maintained on ice for
15 minutes. The homogenate was then transferred to a
thick-walled 50 ml polypropylene centrifuged tube
(Fisher Scientific Company, Pittsburg, PA). The
solution was centrifuged at 10,000 x g for 20 minutes
at 4°C. The upper RNA-containing aqueous layer was
transferred to a fresh 50 ml polypropylene centrifuge
tube and mixed with an equal volume of isopropyl
alcohol. This solution was maintained at -20°C for at
least one hour to precipitate the RNA. The solution
containing the precipitated RNA was centrifuged at
10,000 x g for twenty minutes at 4°C. The pelleted
total cellular RNA was collected and dissolved in 3 ml
of the denaturing solution described above. Three ml

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of isopropyl alcohol was added to the re-suspended
total cellular RNA and vigorously mixed. This
solution was maintained at -20°C for at least 1 hour
to precipitate the RNA. The solution containing the
precipitated RNA was centrifuged at 10,000 x g for ten
minutes at 4°C. The pelleted RNA was washed once with
a solution containing 75% ethanol. The pelleted RNA
was dried under vacuum for 15 minutes and then re-
suspended in dimethyl pyrocarbonate (DEPC) treated
(DEPC-H$_2$O) H$_2$O.

Messenger RNA (mRNA) enriched for sequences
containing long poly A tracts was prepared from the
total cellular RNA using methods described in
<u>Molecular Cloning: A Laboratory Manual</u>, Maniatis et
al., eds., Cold Spring Harbor, NY, (1982). Briefly,
one half of the total RNA isolated from a single
immunized mouse spleen prepared as described above was
resuspended in one ml of DEPC-H$_2$O and maintained at
65°C for five minutes. One ml of 2x high salt loading
buffer consisting of 100 mM Tris-HCl (Tris
[hydroxymethyl] aminomethane hydrochloride), 1 M
sodium chloride (NaCl), 2.0 mM disodium ethylene
diamine tetraacetic acid (EDTA) at pH 7.5, and 0.2%
sodium dodecyl sulfate (SDS) was added to the re-
suspended RNA and the mixture allowed to cool to room
temperature. The mixture was then applied to an
oligo-dT (Collaborative Research Type 2 or Type 3)
column that was previously prepared by washing the
oligo-dT with a solution containing 0.1 M sodium
hydroxide and 5 mM EDTA and then equilibrating the
column with DEPC-H$_2$O. The eluate was collected in a
sterile polypropylene tube and reapplied to the same
column after heating the eluate for 5 minutes at 65°C.
The oligo-dT column was then washed with 2 ml of high
salt loading buffer consisting of 50 mM Tris-HCl, pH

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7.5, 500 mM sodium chloride, 1 mM EDTA at pH 7.5 and
0.1% SDS. The oligo dT column was then washed with 2
ml of 1 X medium salt buffer consisting of 50 mM Tris-
HCl, pH 7.5, 100 mM, 1 mM EDTA and 0.1% SDS. The
5   messenger RNA was eluted from the oligo-dT column with
1 ml of buffer consisting of 10 mM Tris-HCl, pH 7.5, 1
mM EDTA, at pH 7.5, and 0.05% SDS. The messenger RNA
was purified by extracting this solution with phenol/
chloroform followed by a single extraction with 100%
10   chloroform. The messenger RNA was concentrated by
ethanol precipitation and resuspended in DEPC H_2O.

The messenger RNA (mRNA) isolated by the above
process contains a plurality of different V_H coding
polynucleotides, i.e., greater than about 10^6
15   different V_H-coding genes, and contains a similar
number of V_L-coding genes. Thus, the mRNA population
represents a repertoire of variable region-coding
genes.

c.   Preparation of DNA Homologs

20   In preparation for PCR amplification, mRNA
prepared above is used as a template for cDNA
synthesis by a primer extension reaction. In a
typical 50 μl transcription reaction, 5-10 μg of
spleen mRNA in water is first hybridized (annealed)
25   with 500 ng (50.0 pmol) of the 3' V_H primer (primer
12A, Table 5), at 65C for five minutes. Subsequently,
the mixture is adjusted to 1.5 mM dATP, dCTP, dGTP and
dTTP, 40 mM Tris-HCl, pH 8.0, 8 mM $MgCl_2$, 50 mM NaCl,
and 2 mM spermidine. Moloney-Murine Leukemia virus
30   Reverse transcriptase (Stratagene), 26 units, is added
and the solution is maintained for 1 hour at 37°C.

PCR amplification is performed in a 100 μl
reaction containing the products of the reverse
transcription reaction (approximately 5 ug of the
35   cDNA/RNA hybrid), 300 ng of 3' V_H primer (primer 12A

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of Table 5), 300 ng each of the 5' V$_H$ primers
(primers 2-10 of Table 5) 200 mM of a mixture of
dNTP's, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 15 mM MgCl$_2$,
0.1% gelatin and 2 units of <u>Thermus aquaticus</u> (Taq)
DNA polymerase (Perkin-Elmer-Cetus, Emeryville,
California). The reaction mixture is overlaid with
mineral oil and subjected to 40 cycles of
amplification. Each amplification cycle includes
denaturation at 92°C for 1 minute, annealing at 52°C
for 2 minutes and polynucleotide synthesis by primer
extension (elongation) at 72°C for 1.5 minutes. The
amplified V$_H$-coding DNA homolog containing samples are
then extracted twice with phenol/chloroform, once with
chloroform, ethanol precipitated and are stored at -
70°C in 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA.

Using unique 5' primers (2-9, Table 5), efficient
V$_H$-coding DNA homolog synthesis and amplification from
the spleen mRNA is achieved as shown by agarose gel
electrophoresis. The amplified cDNA (V$_H$-coding DNA
homolog) was seen as a major band of the expected size
(360 bp). The amount the amplified V$_H$-coding
polynucleotide fragment in each reaction is similar,
indicating that all of these primers were about
equally efficient in initiating amplification. The
yield and quality of the amplification with these
primers is reproducible.

The primer containing inosine also synthesizes
amplified V$_H$-coding DNA homologs from spleen mRNA
reproducibly, leading to the production of the
expected sized fragment, of an intensity similar to
that of the other amplified cDNAs. The presence of
inosine also permits efficient DNA homolog synthesis
and amplification, clearly indicating that such
primers are useful in generating a plurality of V$_H$-
coding DNA homologs. Amplification products obtained

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from the constant region primers (primers 11 and 13, Table 5) are more intense indicating that amplification was more efficient, possibly because of a higher degree of homology between the template and
5   primers. Following the above procedures, a V_H-coding gene library is constructed from the products of eight amplifications, each performed with a different 5' primer. Equal portions of the products from each primer extension reaction are mixed and the mixed
10   product is then used to generate a library of V_H-coding DNA homolog-containing vectors.

DNA homologs of the V_L are also prepared from the purified mRNA prepared as described above. In preparation for PCR amplification, mRNA prepared
15   according to the above examples is used as a template for cDNA synthesis. In a typical 50 μl transcription reaction, 5-10 μg of spleen mRNA in water is first annealed with 300 ng (50.0 pmol) of the 3' V_L primer (primer 14, Table 5), at 65°C for five minutes.
20   Subsequently, the mixture is adjusted to 1.5 mM dATP, dCTP, dGTP, and dTTP, 40 mM Tris-HCl, pH 8.0, 8 mM $MgCl_2$, 50 mM NaCl, and 2 mM spermidine. Moloney-Murine Leukemia virus reverse transcriptase (Stratagene), 26 units, is added and the solution is
25   maintained for 1 hour at 37°C. The PCR amplification is performed in a 100 μl reaction containing approximately 5 μg of the cDNA/RNA hybrid produced as described above, 300 ng of the 3' V_L primer (primer 14 of Table 5), 300 ng of the 5' V_L primer (primer 16 of
30   Table 5), 200 mM of a mixture of dNTP's, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 0.1% gelatin and 2 units of Taq DNA polymerase. The reaction mixture is overlaid with mineral oil and subjected to 40 cycles of amplification. Each amplification cycle includes
35   denaturation at 92°C for 1 minute, annealing at 52°C

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for 2 minutes and elongation at 72°C for 1.5 minutes.
The amplified samples are then extracted twice with
phenol/chloroform, once with chloroform, ethanol
precipitated and are stored at -70°C in 10 mM Tris-
HCl, 7.5 and 1 mM EDTA.

    d.   Insertion of DNA Homologs into a DNA
          Expression Vector

          To prepare an expression library enriched in
V_H sequences, DNA homologs enriched in V_H sequences
are prepared according to Example 2c using the same
set of 5' primers but with primer 12A (Table 5) as the
3' primer. The resulting PCR amplified products (2.5
μg/30 μl of 150 mM NaCl, 8 mM Tris-HCl, pH 7.5, 6 mM
$MgSO_4$, 1 mM DTT, 200 μg/ml BSA) are digested at 37C
with restriction enzymes Xho I (125 units) and Spe I
(125 units). In cloning experiments which required a
mixture of the products of the amplification
reactions, equal volumes (50 μl, 1-10 μg
concentration) of each reaction mixture are combined
after amplification but before restriction digestion.
The V_H homologs are purified on a 1% agarose gel using
the standard electroelution technique described in
Molecular Cloning A Laboratory Manual, Maniatis et
al., eds., Cold Spring Harbor, NY, (1982). After gel
electrophoresis of the digested PCR amplified spleen
mRNA, the region of the gel containing DNA fragments
of approximate 350 bp is excised, electroeluted into a
dialysis membrane, ethanol precipitated and
resuspended in a TE solution containing 10 mM Tris-
HCl, pH 7.5 and 1 mM EDTA to a final concentration of
50 ng/μl. The resulting V_H DNA homologs represent a
repertoire of polypeptide genes having cohesive
termini adapted for directional ligation to the vector
Lambda Hc2. These prepared V_H DNA homologs are then
directly inserted by directional ligation into

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linearized Lambda Hc2 expression vector prepared as
described below.

The Lambda Hc2 expression DNA vector is prepared
for inserting a DNA homolog by admixing 100 μg of this

5   DNA to a solution containing 250 units each of the
restriction endonucleases Xho I and Spe I (both from
Boehringer Mannheim, Indianapolis, IN) and a buffer
recommended by the manufacturer. This solution is
maintained at 37 from 1.5 hours. The solution is

10   heated at 65°C for 15 minutes top inactivate the
restriction endonucleases. The solution is chilled to
30°C and 25 units of heat-killable (HK) phosphatase
(Epicenter, Madison, WI) and $CaCl_2$ is admixed to it
according to the manufacturer's specifications. This

15   solution is maintained at 30°C for 1 hour. The DNA is
purified by extracting the solution with a mixture of
phenol and chloroform followed by ethanol
precipitation. The Lambda Hc2 expression vector is
now ready for ligation to the V_H DNA homologs prepared

20   in the above examples. These prepared V_H DNA homologs
are then directly inserted into the Xho I and Spe I
restriction digested Lambda Hc2 expression vector that
prepared above by ligating 3 moles of V_H DNA homolog
inserts with each mole of the Hc2 expression vector

25   overnight at 5°C. Approximately 3.0×10^5 plague
forming units are obtained after packaging the DNA
with Gigapack II Bold (Stratagene) of which 50% are
recombinants. The ligation mixture containing the V_H
DNA homologs are packaged according to the

30   manufacturers specifications using Gigapack Gold II
Packing Extract (Stratagene). The resulting Lambda
Hc2 expression libraries are then transformed into
XL1-Blue cells.

To prepare a library enriched in V_L sequences,

35   PCR amplified products enriched in V_L sequences are

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prepared according to Example 2c. These V$_L$ DNA
homologs are digested with restriction enzymes Sac I
and Xba I and the digested V$_L$ DNA homologs are
purified on a 1% agarose gel as described above for
the V$_H$ DNA homologs to form a repertoire of V$_L$-
polypeptide genes adapted for directional ligation.
The prepared V$_L$ DNA homologs are then directionally
ligated into the Lambda Lc2 expression vector
previously digested with the restriction enzymes, Sac
I and Xba I as described for Lambda Hc2. The ligation
mixture containing the V$_L$ DNA homologs is packaged to
form a Lambda Lc2 expression library as described
above and is ready to be plated on XL1-Blue cells.

     e.   <u>Randomly Combining V$_H$ and V$_L$ DNA Homologs on</u>
         <u>the Same Expression Vector</u>

The construction of a library containing
vectors for expressing two cistrons that express heavy
and light chains is accomplished in two steps. In the
first step, separate heavy and light chain libraries
are constructed in the expression vectors Lambda Hc2
and Lambda Lc2, respectively, as described using gene
repertoires obtained from a mouse immunized with NPN-
KLH. In the second step, these two libraries are
combined at the antisymmetric EcoR I sites present in
each vector. This resulted in a library of clones
each of which potentially co-expresses a heavy and a
light chain. The actual combinations are random and
do not necessarily reflect the combinations present in
the B-cell population in the parent animal.

The spleen mRNA resulting from the above
immunizations (Example 2b) is isolated and used to
create a primary library of V$_H$ gene sequences using
the Lambda Hc2 expression vector. The primary library
contains 1.3 x 10^6 plaque-forming units (pfu) and can
be screened for the expression of the decapeptide tag

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to determine the percentage of clones expressing V_H and C_H1 (Fd) sequences. The sequence for this peptide is only in frame for expression following the cloning of a Fd (or V_H) fragment into the vector. At least 80% of the clones in the library express Fd fragments based on immunodetection of the decapeptide tag.

The light chain library is constructed in the same way as the heavy chain and contains 2.5×10^6 members. Plaque screening, using an anti-kappa chain antibody, indicates that 60% of the library contained express light chain inserts. A small percentage of inserts results from incomplete dephosphorylation of vector after cleavage with Sac I and Xba I.

Once obtained, the two libraries are used to construct a combinatorial library by crossing them at the EcoR I site. To accomplish the cross, DNA is first purified from each library.

The Lambda Lc2 library prepared in Example 2d is amplified and 500 μg of Lambda Lc2 expression library phage DNA is prepared from the amplified phage stock using the procedures described in <u>Molecular Cloning:</u> <u>A Laboratory Manual</u>, Maniatis et al., eds., Cold Spring Harbor, NY (1982). Fifty μg of this amplified expression library phage DNA is maintained in a solution containing 100 units of MLu I restriction endonuclease (Boehringer Mannheim, Indianapolis, IN) in 200 μl of a buffer supplied by the endonuclease manufacturer for 1.5 hours at 37°C. The solution is then extracted with a mixture of phenol and chloroform. The DNA is then ethanol precipitated and resuspended in 100 μl of water. This solution is admixed with 100 units of the restriction endonuclease EcoR I (Boehringer) in a final volume of 200 μl of buffer containing the components specified by the manufacturer. This solution is maintained at 37°C for

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1.5 hours and the solution is then extracted with a
mixture of phenol and chloroform. The DNA was ethanol
precipitated and the DNA resuspended in TE.

The Lambda Hc2 expression library prepared in
Example 2d is amplified and 500 μg of Lambda Hc2
expression library phage DNA is prepared using the
methods detailed above. 50 μg of this amplified
library phage DNA is maintained in a solution
containing 100 units of Hind III restriction
endonuclease (Boehringer) in 200 μl of a buffer
supplied by the endonuclease manufacturer for 1.5
hours at 37°C. The solution is then extracted with a
mixture of phenol and chloroform saturated with 0.1 M
Tris-HCl, pH 7.5. The DNA is then ethanol
precipitated and re-suspended in 100 μl of water.
This solution is admixed with 100 units of the
restriction endonuclease EcoR I (Boehringer) in a
final volume of 200 μl of buffer containing the
components specified by the manufacturer. This
solution is maintained at 37°C for 1.5 hours and the
solution is then extracted with a mixture of phenol
and chloroform. The DNA is ethanol precipitated and
the DNA re-suspended in TE.

The restriction digested Hc2 and Lc2 expression
libraries are ligated together. To that end, a DNA
admixture consists of 1 μg of Hc2 and 1 μg of Lc2
phage library DNA is prepared in a 10 μl reaction
using the reagents supplied in a ligation kit
(Stratagene). The DNA admixture is warmed to 45°C for
5 minutes to melt any cohesive termini that may have
reannealed. The admixture is then chilled to 0°C to
prevent religation. Bacteriophage T4 DNA ligase (0.1
Weiss units which is equivalent to 0.02 units as
determined in an exonuclease resistance assay) is
admixed into the chilled DNA solution along with 1 μl

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of 5 mM ATP and 1 μl 10X bacteriophage T4 DNA ligase
buffer (10X buffer is prepared by admixing 200 mM
Tris-HCl, pH 7.6, 50 mM MgCl$_2$, 50 mM DTT, and 500
μg/ml BSA) to form a ligation admixture. After
5   ligation for 16 hr at 4°C, 1 μl of the ligated the
phage DNA is packaged with Gigapack Gold II packaging
extract and plated on XL1-Blue cells prepared
according to the manufacturers instructions to form a
Lambda phage library of dicistronic expression vectors
10   capable of expressing heavy and light chains derived
from the NPN-immunized mouse. A portion of the clones
obtained are used to determine the effectiveness of
the combination.

     f.   Selection of Anti-NPN Reactive Heterodimer-
15   Producing Dicistronic Vectors

     The combinatorial Fab expression library
prepared above in Example 2a was screened to identify
clones having affinity for NPN. To determine the
frequency of the phage clones which co-expressed the
20   light and heavy chain fragments, duplicate lifts of
the light chain, heavy chain and combinatorial
libraries were screened as above for light and heavy
chain expression. In this study of approximately 500
recombinant phage, approximately 60% co-expressed
25   light and heavy chain proteins.

     All three libraries, the light chain, the heavy
chain and the combinatorial, were screened to
determine if they contained recombinant phage that
expressed antibody fragments which bound NPN. In a
30   typical procedure 30,000 phage were plated on XL1-Blue
cells and duplicate lifts with nitrocellulose were
screened for binding to NPN coupled to ^{125}I labeled
BSA. The BSA was iodinated following the Chloramine-T
method as described by Bolton et al., _Biochem._,
35   133:529-534 (1973). Duplicate screens of 80,000

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recombinant phage from the light chain library and a similar number from the heavy chain library did not identify any clones which bound the antigen. In contrast, the screen of a similar number of clones from the Fab expression library identified many phage plaques that bound NPN. This observation indicates that under conditions where many heavy chains in combination with light chains bind to antigen the same heavy or light chains alone do not. Therefore, in the case of NPN, it is believed that there are many heavy and light chains that only bind antigen when they are combined with specific light and heavy chains, respectively.

To assess the ability to screen large numbers of clones and obtain a more quantitative estimate of the frequency of antigen binding clones in the combinatorial library, one million phage plaques were screened and approximately 100 clones which bound to antigen were identified. For six clones which were believed to bind NPN, a region of the plate containing the six positive and approximately 20 surrounding bacteriophage plaques was selected and each plaque was cored, replated, and screened with duplicate lifts. As expected, approximately one in twenty of the phage specifically bound to antigen. Cores of regions of the plated phage believed to be negative did not give positives on replating.

Clone 2b, one of the plaques which reacted with NPN, was excised according to an _in vivo_ excision protocol where 200 μl of phage stock and 200 μl of a F+ derivative of XL1-Blue (A_{600} = 1.00) (Stratagene) were admixed with 1 μl of M13mp8 helper phage (1 X 10^{10} pfu/milliliter (ml)) and maintained at 37°C for 15 minutes. After a four hour maintenance in Luria-Bertani (LB) medium and heating at 70°C for 20 minutes

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to heat kill the XL1-Blue cells, the phagemids were
re-infected into XL1-Blue cells and plated onto LB
plates containing ampicillin. This procedure
converted the cloned insert from the Lambda Zap II
5   vector into a plasmid vector to allow easy
manipulation and sequencing (Stratagene). The
phagemid DNA encoding the V_H and part of the V_L was
then determined by DNA sequencing using the Sanger
dideoxy method described in Sanger et al., <u>Proc. Natl.</u>
10   <u>Acad. Sci.</u>, 74:5463-5467 (1977) using a Sequenase kit
according to manufacturer's instructions (US
Biochemical Corp., Cleveland, Ohio). The nucleotide
residue sequence of Clone 2b Fd chain is listed in the
Sequence Listing as SEQ ID NO 99. The nucleotide
15   residue sequences of the kappa light chain variable
and constant regions are listed in the Sequence
Listing as SEQ ID NO 100 and SEQ ID NO 101,
respectively.

g.   <u>Preparation of a DNA Sequence Encoding a</u>
20   <u>Filamentous Phage Coat Protein Membrane</u>
          <u>Anchor</u>

          <u>cpVIII Membrane Anchor</u>: M13mp18, a
commerically available bacteriophage vector
(Pharmacia, Piscataway, New Jersey), was used as a
25   source for isolating the gene encoding cpVIII. The
sequence of the gene encoding the membrane anchor
domain of cpVIII listed in Sequence Listing as SEQ ID
NO 102, was modified through PCR amplification to
incorporate the restriction endonuclease sites, Spe I
30   and EcoR I, and two stop codons prior to the EcoR I
site. The corresponding amino acid residue sequence
of the membrane anchor domain of cpVIII is listed as
SEQ ID NO 17.

     To prepare a modified cpVIII, replicative form
35   DNA from M13mp18 was first isolated. Briefly, into 2

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ml of LB (Luria-Bertani medium), 50 μl of a culture of
a bacterial strain carrying an F' episome (JM107,
JM109 or TG1) was admixed with a one tenth suspension
of bacteriophage particles derived from a single
5   plaque. The admixture was incubated for 4 to 5 hours
at 37°C with constant agitation. The admixture was
then centrifuged at 12,000 x g for 5 minutes to pellet
the infected bacteria. After the supernatant was
removed, the pellet was resuspended by vigorous
10   vortexing in 100 μl of ice-cold solution I. Solution
I was prepared by admixing 50 mM glucose, 10 mM EDTA
and 25 mM Tris-HCl, pH 8.0, and autoclaving for 15
minutes.

    To the bacterial suspension, 200 μl of freshly
15   prepared Solution II was admixed and the tube was
rapidly inverted five times. Solution II was prepared
by admixing 0.2 N NaOH and 1% SDS. To the bacterial
suspension, 150 μl of ice-cold Solution III was
admixed and the tube was vortexed gently in an
20   inverted position for 10 seconds to disperse Solution
III through the viscous bacterial lysate. Solution
III was prepared by admixing 60 ml of 5 M potassium
acetate, 11.5 ml of glacial acetic acid and 28.5 ml of
water. The resultant bacterial lysate was then stored
25   on ice for 5 minutes followed by centrifugation at
12,000 x g for 5 minutes at 4°C in a microfuge. The
resultant supernatant was recovered and transferred to
a new tube. To the supernatant was added an equal
volume of phenolchloroform and the admixture was
30   vortexed. The admixture was then centrifuged at
12,000 x g for 2 minutes in a microfuge. The
resultant supernatant was transferred to a new tube
and the double-stranded bacteriophage DNA was
precipitated with 2 volumes of ethanol at room
35   temperature. After allowing the admixture to stand at

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room temperature for 2 minutes, the admixture was
centrifuged to pellet the DNA. The supernatant was
removed and the pelleted replicative form DNA was
resuspended in 25 μl of Tris-HCl, pH 7.6, and 10 mM
5   EDTA (TE).

The double-stranded M13mp18 replicative form DNA
was then used as a template for PCR. Primers, AK 5
(SEQ ID NO 103) and AK 6 (SEQ ID NO 104), the
sequences of which are listed in Table 7 below, were
10   used in the PCR reaction to amplify the mature gene
for cpVIII member anchor domain and incorporate the
two cloning sites, Spe I and EcoR I. For the PCR
reaction, 2 μl containing 1 ng of M13mp18 replicative
form DNA was admixed with 10 μl of 10X PCR buffer
15   purchased commercially (Promega Biotech, Madison,
Wisconsin) in a 0.5 ml microfuge tube. To the DNA
admixture, 8 μl of a 2.5 mM solution of dNTPs (dATP,
dCTP, dGTP, dTTP) was admixed to result in a final
concentration of 200 micromolar (uM). Three μl
20   (equivalent to 60 picomoles (pM)) of the 5' forward AK
5 primer and 3 μl (60 pM) of the 3' backward AK 6
primer was admixed into the DNA solution. To the
admixture, 73 μl of sterile water and 1 μl/5 units of
polymerase (Promega Biotech) was added. Two drops of
25   mineral oil were placed on top of the admixture and 40
rounds of PCR amplification in a thermocycler were
performed. The amplification cycle consisted of 52°C
for 2 minutes, 72°C for 1.5 minutes and 91°C for 2
minutes. The resultant PCR modified cpVIII membrane
30   anchor domain DNA fragment from M13mp18 containing
samples were then purified with Gene Clean (BIO101, La
Jolla, California), extracted twice with
phenol/chloroform, once with chloroform followed by
ethanol precipitation and were stored at -70°C in 10
35   mM Tris-HCl, pH 7.5, and 1 mM EDTA.

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Table 7

```
SEQ.
ID. NO.    Primer
(103)¹    AK 5  (F)  5' GTGCCCAGGGATTGTACTAGTGCTGAGGGTGACGAT 3'
(104)²    AK 6  (B)  5' ACTCGAATTCTATCAGCTTGCTTTCGAGGTGAA 3'
(105)³    Hc3   (F)  5' AGGTCCAGCTTCTCGAGTCTGG 3'
(106)⁴    AK 7  (B)  5' GTCACCCTCAGCACTAGTACAATCCCTGGGCAC 3'
(107)⁵    G-3   (F)  5' GAGACGACTAGTGGTGGCGGTGGCTCTCCATTC
                       GTTTGTGAATATCAA 3'
(108)⁶    G-3   (B)  5' TTACTAGCTAGCATAATAACGGAATACCCAAAA
                       GAACTGG 3'
(109)⁷    LAC-F      5' TATGCTAGCTAGTAACACGACAGGTTTCCCGAC
                       TGG 3'
(110)⁸    LAC-B      5' ACCGAGCTCGAATTCGTAATCATGGTC 3'
(125)9    LAC-B'     5' AGCTGTTGAATTCGTGAAATTGTTATCCGCT 3'
```

F Forward Primer

B Backward Primer

1 From 5' to 3': the overlapping sequence for C_H1 3'
   end is double underlined; the Spe I restriction
   site sequence is single underlined; the overlapping
   sequence for cpVIII is double underlined.

2 EcoR I restriction site sequence is single
   underlined

3 Xho I restriction site sequence is underlined

4 From 5' to 3': the overlapping sequence for cpVIII
   is double underlined; the Spe I restriction site
   sequence is single underlined; the overlapping
   sequence for C_H1 3' end is double underlined.

5 From 5' to 3': Spe I restriction site sequence is
   single underlined; the overlapping sequence with
   the 5' end of cpIII is double underlined

6 From 5' to 3': Nhe I restriction site sequence is
   single underlined; the overlapping sequence with 3'

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end of cpIII is double underlined.

7 From 5' to 3': overlapping sequence with the 3'
   end of cpIII is double underlined; Nhe I
   restriction sequence begins with the nucleotide
5   residue "G" at position 4 and extends 5 more
   residues = GCTAGC.

8 EcoR I restriction site sequence is single
   underlined.

9. Alternative backwards primer for amplifying LacZ;
10 EcoR I restriction site sequence is single
   underlined.

_____

To verify amplification of the modified cpVIII
15 membrane anchor domain, the PCR purified DNA products
were electrophoresed in a 1% agarose gel. The
expected size of the cpVIII was approximately 150 base
pairs. The area in the agarose containing the
modified cpVIII DNA fragment was isolated from the
20 agarose as described above. The sequence of the
isolated modified cpVIII DNA fragment is listed as SEQ
ID NO 111. The isolated cpVIII DNA fragment was then
admixed with a similarly prepared fragment of modified
Fd as described below in Example 2i in order to form a
25 DNA segment encoding the fusion protein Fd-cpVIII.

cpIII Membrane Anchor: M13mp18 was also used as
a source for isolating the gene encoding the membrane
anchor domain at cpIII, the sequence of which is
listed in the Sequence Listing as SEQ ID NO 112. The
30 amino acid residue sequence of membrane anchor domain
cpIII is listed in SEQ ID NO 16. M13mp18 replicative
form DNA was prepared as described above and used as a
template for two PCR amplifications for construction
of a DNA fragment consisting of the mature gene for
35 cpIII membrane anchor domain located 5' to a sequence

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encoding the LacZ promoter, operator and cap-binding
site for controlling light chain expression. The
restriction sites, Spe I and EcoR I, were created in
the amplification reactions and were located at the 5'
and 3' ends of the fragment respectively. The
procedure for creating this fragment by combining the
products of two separate PCR amplifications is
described below.

The primer pair, G-3(F) (SEQ ID NO 107) and G-
3(B) (SEQ ID NO 108) listed in Table 7, was used in
the first PCR reaction as performed above to amplify
the cpIII membrane anchor gene and incorporate Spe I
and Nhe I restriction sites into the fragment. The
amplified PCR fragment also contained nucleotide
sequences for encoding a five amino acid tether
composed of four glycerine residues and one serine
juxtaposed between the heavy chain and cpIII encoding
domains. Once expressed, the five amino acid sequence
lacking an orderly secondary structure served to
minimize the interaction between the Fab and cpIII
domains. The resultant PCR modified cpIII DNA
fragment having Spe I and Nhe I sites in the 5' and 3'
ends, respectively, of the fragment was verified and
purified as described above. The sequence of the PCR
modified cpIII membrane anchor domain DNA fragment is
listed in the Sequence Listing as SEQ ID NO 113. A
second PCR amplification using the primer pairs, Lac-F
(SEQ ID NO 109) and Lac-B (SEQ ID NO 110) listed in
Table 7, was performed on a separate aliquot of
M13mp18 replicative form template DNA to amplify the
LacZ promoter, operator and Cap-binding site having a
5' Nhe I site and a 3' EcoR I site. The primers used
for this amplification were designed to incorporate a
Nhe I site on the 5' end of the amplified fragment to
overlap with a portion of the 3' end of the cpIII gene

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fragment and of the Nhe I site 3' to the amplified
cpIII fragment. The reaction and purification of the
PCR product was performed as described above. The
sequence of the resultant PCR modified cpIII DNA
5   fragment having a 5' Nhe I and 3' EcoR I restriction
site is listed in the Sequence Listing as SEQ ID NO
114.

An alternative Lac-B primer for use in
constructing the cpIII membrane anchor and LacZ
10   promotor region was Lac-B' as shown in Table 7. The
amplification reactions were performed as described
above with the exception that in the second PCR
amplification, Lac-B' was used with Lac-F instead of
Lac-B. The product from the amplification reaction is
15   listed in the sequence listing as SEQ ID NO 114 from
nucleotide position 1 to nucleotide position 172. The
use of Lac-B' resulted in a LacZ region lacking 29
nucleotides on the 3' end but was functionally
equivalent to the longer fragment produced with the
20   Lac-F and Lac-B primers.

The products of the first and second PCR
amplifications using the primer pairs 6-3(F) and 6-
3(B) and Lac-F and Lac-B were then recombined at the
nucleotides corresponding to cpIII membrane anchor
25   overlap and Nhe I restriction site and subjected to a
second round of PCR using the G3-F (SEQ ID NO 107) and
Lac-B (SEQ ID NO 110) primer pair to form a recombined
PCR DNA fragment product consisting of the following:
a 5' Spe I restriction site; a cpIII DNA membrane
30   anchor domain beginning at the nucleotide residue
sequence which corresponds to the amino acid residue
198 of the entire mature cpIII protein; an endogenous
stop site provided by the membrane anchor at amino
acid residue number 112; a Nhe I restriction site, a
35   LacZ promoter, operator and Cap-binding site sequence;

and a 3' EcoR I restriction site. The recombined PCR
modified cpIII membrane anchor domain DNA fragment was
then restriction digested with Spe I and EcoR I to
produce a DNA fragment for directional ligation into a
5   pComb2 phagemid expression vector having only one Spe
I site prepared in Example 1a(iv) to form a pComb2-III
(also referred to as pComb2-III) phagemid expression
vector as described in Example 1b(ii).

    h.   Isolation of Anti-NPN Coding V_H DNA Segment
10   To prepare modified Fd fragments for
recombination with the PCR modified cpVIII membrane
anchor domain fragment to form a Fd-cpVIII DNA fusion
product, PCR amplification as described above was
performed using Clone 2b, prepared in Example 2f, as a
15   template. The primers, Hc3 (SEQ ID NO 105) and AK 7
(SEQ ID NO 106), the sequences of which are listed in
Table 7, were used in PCR to amplify the Fd portion of
the Clone 2b and incorporate Xho I and Spe I cloning
sites along with a cpVIII overlapping sequence. The
20   amplified PCR modified Fd product was purified,
electrophoresed and isolated from 1% agarose gels as
described above. The size of the Fd fragment was 680
base pairs.

    i.   Preparation of a DNA Segment Encoding a
25   Portion of the Fusion Protein Fd-cpVIII
          The purified PCR modified Fd DNA fragment
containing cpVIII overlapping nucleotide sequences
prepared above was then admixed with the PCR modified
cpVIII membrane anchor domain fragment to form an
30   admixture. The fragments in the admixture were
allowed to recombine at their complementary regions.
The admixture containing the recombined PCR fragments
was then subjected to a second round of PCR
amplification as described above using the end primer
35   pair AK 6 (SEQ ID NO 104) and Hc3 (SEQ ID NO 105)

(Table 7). The corresponding product of the PCR
amplification was purified and electrophoresed on
agarose gels as described above. The PCR product was
determined to be approximately 830 base pairs (Fd =

5   680 + 150) confirming the fusion of Fd with cpVIII.
The sequence of the PCR product linking the Fd
sequence with the cpVIII sequence in frame in a 5' to
3' direction is listed as SEQ ID NO 115. The Fd-
cpVIII fusion product was then used in directional

10 ligations described in Example 2j for the construction
of a pCBAK8-2b dicistronic phagemid expression vector.

     j.   Construction of pCBAK8-2b Dicistronic
        Expression Vector

        To construct a phagemid vector for the

15 coordinate expression of a Fd-cpVIII fusion protein
with kappa light chain, the PCR amplified Fd-cpVIII
fusion product prepared in above in Example 2i was
first ligated into Clone 2b phagemid expression vector
isolated from the NPN combinatorial library prepared

20 in Example 2f. For the ligation, the Fd-cpVIII PCR
fusion product was first restriction digested with Xho
I and EcoR I. Clone 2b phagemid vector was similarly
digested resulting in the removal of the cloning and
decapeptide regions. The digested Fd-cpVIII fragment

25 was admixed and ligated into the digested Clone 2b at
the cohesive termini generated by Xho I and EcoR I
restriction digestion. The ligation resulted in
operatively linking the nucleotide residue sequence
encoding the Fd-cpVIII polypeptide fusion protein to a

30 second cassette having the nucleotide residue
sequences encoding the ribosome binding site, a pelB
leader sequence and the kappa light chain already
present in Clone 2b to form a dicistronic DNA molecule
in the original Clone 2b phagemid expression vector.

35   E. coli, strain TG1, was then transformed with

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the phagemid containing the dicistronic DNA molecule
and transformants were selected on ampicillin as the
original Clone 2b contained an ampicillin selectable
resistance marker gene. For high efficiency electro-
transformation of E. coli, a 1:100 volume of an
overnight culture of TG1 cells was inoculated into one
liter of L-broth (1% Bacto tryptone, 0.5% Bacto yeast
extract, 0.5% NaCl). The cell suspension was
maintained at 37C with vigorous shaking to a
absorbance at 600 nm of 0.5 to 1.0. The cell
suspension in log phase growth was then harvested by
first chilling the flask on ice for 15 to 30 minutes
followed by centrifugation in a cold rotor at 4000 x g
for 15 minutes to pellet the bacteria. The resultant
supernatant was removed and the bacterial cell pellet
was resuspended in a total of one liter of cold water
to form a cell suspension. The centrifugation and
resuspension procedure was repeated two more times and
after the final centrifugation, the cell pellet was
resuspended in 20 ml of cold 10% glycerol. The
resuspended cell suspension was then centrifuged to
form a cell pellet. The resultant cell pellet was
resuspended to a final volume of 2 to 3 ml in cold 10%
glycerol resulting in a cell concentration of 1 to 3 X
10^{10} cells/ml. For the electro-transformation
procedure, 40 μl of the prepared cell suspension was
admixed with 1 to 2 μl of phagemid DNA to form a cell-
phagemid DNA admixture. The resultant admixture was
mixed and allowed to sit on ice for one minute. An
electroporation apparatus, for example a Gene Pulsar,
was set a 25 uF and 2.5 kV. The pulse controller was
set to 200 ohms. The cell-DNA admixture was
transferred to a cold 0.2 cm electroporation cuvette.
The cuvette was then placed in the chilled safety
chamber and pulsed once at the above settings. To the

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pulsed admixture, 1 ml of SOC medium was then admixed
and the cells were resuspended with a Pasteur pipette
(SOC medium was prepared by admixing 2% Bacto
tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM
KCl, 10 mM MgCl$_2$, 10 mM MgSO$_4$, and 20 mM glucose).
The cells suspension was then transferred to a 17 X
100 mm polypropylene tube and maintained at 37°C for
one hour. After the maintenance period, the
transformed TG1 cells were then plated on ampicillin
LB plates for selection of ampicillin resistant
colonies containing the phagemid which provided the
selectable marker gene.

Ampicillin resistant colonies were selected and
analyzed for the correct insert size and expression of
Fab. Briefly, DNA minipreps of selected colonies were
prepared for the isolation of phagemid DNA. The
isolated phagemid DNA from each miniprep was
restriction digested with Xho I and EcoR I and the
digests were electrophoresed on a 1% agarose gel.
Clone AK16 was selected as an 830 bp fragment was
visualized on the gels confirming the insertion of the
Fd-cpVIII PCR fusion product into digested Clone 2b.

Clone AK16 phagemid was then restriction digested
with Xho I and Xba I and the nucleotide residue
sequence of the dicistronic DNA molecule encoding the
Fd-cpVIII fusion protein, the ribosome binding site
and pelB leader sequence for expression of the light
chain, a spacer region and the 2b kappa light chain
was isolated by agarose gel electrophoresis. The
isolated dicistronic DNA fragment was then ligated
into a Xho I and Xba I restriction digested pCBAKO
expression vector prepared in Example 1c(ii) to form a
dicistronic phagemid expression vector designated
pCBAK8-2b.

The resultant pCBAK8-2b expression vector

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consisted of nucleotide residue sequences encoding the
following elements: f1 filamentous phage origin of
replication; a chloramphenicol acetyl transferase
selectable resistance marker gene; an inducible LacZ
promoter upstream from the LacZ gene; a multiple
cloning site flanked by T3 and T7 polymerase
promoters; and the dicistronic DNA molecule (a first
cassette consisting of a ribosome binding site, a pelB
leader, and a Fd-cpVIII DNA fusion product operatively
linked to a second cassette consisting of a second
ribosome binding site, a second pelB leader, and a
kappa light chain).

        k.   Construction of pCBAK3-2b Dicistronic
              Expression Vector
        To construct a phagemid vector for the
coordinate expression of a Fd-cpIII fusion protein
with kappa light chain, the PCR amplified and
recombined cpIII membrane anchor and LacZ promotor
region fragment prepared in Example 2g having a 5' Spe
I and 3' EcoR I restriction site was first
directionally ligated into a pComb2 phagemid
expression vector previously digested with Spe I and
EcoR I prepared in Example 1a(iv) to form a pComb2-3
(also called pComb2-III) phagemid vector. See Example
1b(ii) for details of vector construction. This
vector was used in this invention when ampicillin
resistant vectors were preferred. Thus, the resultant
pComb2-3 vector, having only one Spe I restriction
site, contained separate LacZ promoter/operator
sequences for directing the separate expression of the
heavy chain (Fd)-cpIII fusion product and the light
chain protein. The expressed proteins were directed
to the periplasmic space by pelB leader sequences for
functional assembly on the membrane. Inclusion of the

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phage Fl intergenic region in the vector allowed for
packaging of single stranded phagemid with the aid of
helper phage. The use of helper phage superinfection
lead to expression of two form of cpIII. Thus, normal
5   phage morphogenesis was perturbed by competition
between the Fab-cpIII fusion and the native cpIII of
the helper phage for incorporation into the virion as
schematically shown in Figure 8 for Fab-cpVIII
fusions.

10   For producing chloramphenicol resistant vectors
for use in this invention, the resultant pComb2-3
phagemid vector was then restriction digested with Sac
II and Apa I to form an isolated fragment. The
resultant isolated fragment containing the expression
15   control sequences and the cpIII sequence was then
directionally ligated into a similarly digested pCBAKO
phagemid vector prepared in Example 1c(ii) to form a
pCBAK3 phagemid expression vector. This vector lacked
Fd and kappa light chain sequences.

20   A chloramphenicol-resistant phagemid expression
vector, pCBAK3-2b, for the expression of a fusion
protein and kappa light chain was then constructed.
Briefly, the pCBAK3 phagemid expression vector
prepared above was first digested with Xho I and Spe I
25   to form a linearized pCBAK3 phagemid expression
vector. PCR amplified and modified Fd fragment,
prepared in Example 2h containing Xho I and Spe I
sites, was subsequently restriction digested with Xho
I and Spe I. The resultant Fd fragment was then
30   directionally ligated via cohesive termini into the
Xho I and Spe I restriction digested pCBAK3 phagemid
expression vector to form a second phagemid expression
vector in which the PCR modified Fd fragment was
operatively linked in-frame to nucleotide residue
35   sequences encoding cpIII. E. coli strain XL1-Blue

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(Stratagene) was then transformed with the above
phagemid vector containing Fd-cpIII. Transformants
containing the Fd-cpIII encoding phagemid were
selected on chloramphenicol. Phagemid DNA was
isolated from chloramphenicol resistant clones and was
restriction digested with Sac I and Xba I to form a
linearized phagemid expression vector into which a Sac
I and Xba I light chain fragment prepared below was
directionally ligated.

Phagemid Clone 2b, isolated from the original
combinatorial library as described in Example 2a, was
restriction digested with Sac I and Xba I to isolate
the nucleotide residue sequence encoding the kappa
light chain. The isolated kappa light chain sequence
was then directionally ligated into the Sac I and Xba
I restriction digested phagemid expression vector
prepared above containing Fd-cpIII to form the
phagemid expression vector, pCBAK3-2b. The resultant
vector contained the nucleotide residue sequence of a
dicistronic DNA molecule for the coordinate expression
of a Fd-cpIII fusion protein with kappa light chain.
The resultant phagemid expression vector consisted of
nucleotide residue sequences encoding the following
elements: f1 filamentous phage origin of replication;
a chloramphenicol acetyl transferase selectable
resistance marker gene; an inducible LacZ promoter
upstream from the LacZ gene; a multiple cloning site
flanked by T3 and T7 polymerase promoters; and the
dicistronic molecule (a first cassette consisting of a
first ribosome binding site and pelB leader
operatively linked to Fd-cpIII operatively linked to a
second cassette consisting of a second LacZ, a second
ribosome binding site, and a second pelB leader
operatively linked to a kappa light chain).

XL1-Blue cells were then transformed with the

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phagemid expression vector pCBAK3-2b. Transformed
colonies containing the chloramphenicol resistant
phagemids were selected as described above and
analyzed for the correct size insert and expression of
5   Fab as described in Example 2j. Following
verification of the insert and expression of Fab in
the pCBAK3-2b phagemid vector, XL1-Blue cells were
then transformed and induced for the expression of Fab
antibodies as described in Examples 3 and 4.

10   The results of the expression, selection and
screening of the Fab-cpIII fusions revealed an
advantage of monovalent display provided by Fab-cpIII
fusions over multivalent displays provided by
Fab-cpVIII fusions as it allowed for the sorting of
15   clones based on affinity as well as specificity, as
does the immune system. A 253-fold enrichment of the
tight binding clone 10C over the weaker binding clone
7E using the pComb3 system as described in Barbas et
al., _Proc. Natl. Acad. Sci., USA_, 88:7978-7982 (1991).
20   Studies with peptide libraries on phage that displayed
four to five copies of the peptide on the phage
surface have shown that multivalency prevented the
separation of phage displaying moderate affinity
peptides (10^{-6} M) from those displaying high affinity
25   peptides (10^{-9} M). Cwirla et al., _Proc. Natl. Acad._
Sci., USA, 87:6378-6382 (1990). Multivalency lead to
a chelation effect that reduces the ability to
discriminate between phage-bearing high affinity Fabs
from those bearing low affinity Fabs.
30   The use of the system was further demonstrated by
sorting a previously characterized (one binder per
5000 clones) human combinatorial antitetanus toxoid
Fab library as described in Example 6 and by Persson
et al., _Proc. Natl. Acad. Sci., USA_, 88:2432-2436
35   (1991). The library, originally in a lambda phage

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vector system, was reconstructed in pComb2-3 retaining
the original pairings of heavy and light chains. The
library size, 10^7 clones was 10-fold larger than the
original lambda phage library. After a single round

5   of panning, 13 or 57 clones picked were determined to
be tetanus toxoid binders which represented a 10^3-fold
enrichment. Following the third panning, the phage
yield had increased 200-fold, indicating enrichment of
specific phage. All the clones were thus

10   antigen-specific binders. Large combinatorial
libraries of 10^8 members are thus accessible using
this system. Even larger libraries are achieved by
mutagenesis as described in Examples 6-8.

15   3.   Expression of Anti-NPN Heterodimer on Phage
             Surfaces
             For expression of antibody Fab directed against
NPN on phage surfaces, XL1-Blue cells were separately
transformed with the phagemid vectors, pCBAK8-2b and

20   pCBAK3-2b, prepared in Examples 2j and 2k,
respectively. The transformants were selected on LB
plates containing 30 μg/ml chloramphenicol.
Antibiotic resistant colonies were selected for each
phagemid transformation and grown in liquid cultures

25   at 37°C in super broth (super broth was prepared by
admixing the following: 20 g 3 [N-Morpholino] propane-
sulfonic acid (MOPS); 60 g tryptone; 40 g yeast
extract; and 2 liter of water; adjust pH to 7.0 with
10 m NaOH) containing 30 μg/ml chloramphenicol and

30   12.5 μg/ml tetracycline for the respective antibiotic
selection of the phagemid and the F' episome. The
antibiotic resistant transformed XL1-Blue cells were
diluted to an optical density (OD_{600nm}) of 0.4 in super
broth. The inducer, isopropyl thiogalactopyranoside

35   (IPTG), was admixed to the bacterial suspension for a

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final concentration of 1 mM and the admixture was
maintained at 37°C for 1 hour to induce the expression
of the fusion protein and kappa light chain from the
LacZ promoter. Helper phage, either R408 or VCS M13
5   (Stratagene), was then admixed to the induced
bacterial suspension at a ratio of 10-20 helper phage
to 1 transformed bacterial cell to initiate the
generation of copies of the sense strand of the
phagemid DNA. The admixture containing the helper
10   phage was then maintained for an additional two hours
at 37°C to allow for filamentous bacteriophage
assembly wherein the expressed anti-NPN Fab antibodies
fused to either bacteriophage membrane anchor domains
of cpVIII or cpIII were incorporated into surface of
15   the bacteriophage particles. The bacterial suspension
was then centrifuged resulting in a bacterial cell
pellet and a supernatant containing phage. The
supernatant was removed, collected and assayed as
described below for the presence of functional anti-
20   NPN Fab molecules anchored to the phage particles by
either cpVIII or cpIII.

       4.   Assays for Verifying the Presence and Function of
             Anti-NPN Heterodimer on the Surface of
25   Filamentous Phage
             a.   Electron Microscopy
                   To localize functional Fab molecules, the
binding to antigen labelled with colloidal gold was
studied. Phage containing supernatants and bacterial
30   cells prepared in Example 3 were spotted on formvar
(Polysciences, Inc., Warrington, Pennsylvania) coated
grids affixed onto a solid phase. In some experiments
grids were coated with cells and infected with phage
in situ. Subsequently grids were blocked with 1%
35   bovine serum albumin (BSA) in PBS at pH 7.2, washed

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and incubated with 2-7 nanometer (nm) colloidal gold
particles coated with BSA-NPN hapten conjugate for a
time period sufficient to form a labeled
immunoreaction complex. The grids were washed to
remove excess gold particles and negatively stained in
uranylacetate and visualized by electron microscopy.

Examination of filamentous phage and permeablized
cells producing phage revealed specific labelling of
phage or exposed bacterial membranes. Phage were
observed to contain 1 to 24 copies of antigen binding
sites per particle. Neither helper phage alone nor
intact E. coli labelled with antigen. Background
nonspecific binding was very low. Filamentous phage
particles emerging from the E. coli surfaces were
labelled with antigen as shown in Figure 9.

The generation of a related phage surface
expression vector utilizing cpIII as a fusion partner
with Clone 2b, pCBAK3-2b, revealed specific antigen
labelling to the phage head but not the column.
Additionally human anti-tetanus Fab expressed as a
cpIII fusion did not bind to BSA-NPN antigen.

   b. Phage Elisa

       Microtitration plates were coated with NPN-
BSA conjugate (0.1 ml, 1 μg/ml in 0.1 M Tris-HCl, pH
9.2), and blocked with 1% BSA in PBS. Serial two fold
dilutions of pCBAK8-2b derived phage (0.1 ml),
prepared in Example 3, were added to the pre-coated
microtitration plate and incubated for 3 hours at
ambient temperature or 16 hours at 4°C. The plates
were washed with PBS and goat anti-kappa alkaline
phosphatase conjugate (Fisher Biotech, Pittsburgh,
Pennsylvania) added (0.1 ml diluted 1/1000 in PBS
containing 0.1% BSA) and incubated for 2 hours at room
temperature. The plates were washed in PBS and
substrate added (0.1 ml, 1 mg/ml p-

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nitrophenylphosphate in 0.1 M Tris-HCl, pH 9.5,
containing 50 mM $MgCl_2$). After incubation at 37°C for
signal development, the optical densities at 400 nm
were determined. Competition assays were performed
5   with the addition of increasing amounts of free NPN
hapten ranging from zero up to 5 mg/well.

The ELISA assays confirmed the presence of
functional antibody Fab. In a two site ELISA on NPN
antigen coated plates when probed with anti-mouse
10   kappa chain enzyme conjugate, phage supernatant
generated from helper phage infection of cells
carrying the pCBAK8-2b construct exhibited expected
titration curves with serial two fold dilutions of
phage containing antibody. The results of the two-
15   site ELISA are shown in Figure 10. For a signal to be
generated in this assay, the phage particle must (i)
have functionally associated Fd and kappa chains and
(ii) be multivalent. Specificity of the particle was
assessed by inhibiting binding to the plate in the
20   presence of increasing concentrations free hapten.
The generated phage particles exhibited binding to
solid phase of the ELISA and could be inhibited by
addition of hapten as shown in Figure 11. Complete
inhibition was achieved when 5 ng of free NPN hapten
25   was used in the assay. Helper phage did not give a
signal in the ELISA. These results show the
functional assembly of antibody heavy and light chain
polypeptides to form an epitope-binding complex that
is present on the surface of the phage particle and
30   able to bind the preselected ligand chapter containing
an epitope.

       c.   Antigen Specific Precipitation of Phage
              Phage supernatant from XL1-Blue was
transformed with the pCBAK8-2b dicistronic expression
35   vector prepared in Example 3 (1 ml) was incubated with

BSA-NPN conjugate (10 μl, 2 mg/ml) for 18 hours at
4°C. The mixture was then pelleted by centrifugation
at 3000 rpm on a bench top centrifuge and the
appearance of precipitate noted. Helper phage was
5   used as a control. The pellet was washed repeatedly
in cold PBS (5 x 3 ml/wash) and then resuspended in LB
(0.5 ml). The solubilized precipitates were added to
fresh XL1-Blue cells (0.5 ml of overnight culture),
incubated for 1 hour at 37°C and aliquots plated out
10   on LB agar containing chloramphenicol (30 μg/ml).
Colonies were selected randomly. Colony lifts on
nitrocellulose were treated with lysozyme to digest
the cell wall, briefly treated with chloroform to
breakdown the outer membrane, blocked in BSA 1% in PBS
15   and incubated with ^{125}I labelled BSA-NPN antigen.
After several washes in PBS (containing 0.05% Tween-
20), film was exposed to the washed and dried filter
overnight at -70°C and the autoradiographs were then
developed.
20   Precipitates were obtained with antibody
containing phage but not helper phage in the presence
of BSA-NPN. In addition, the particles retained
infectivity on subsequent incubation with bacterial
cells carrying the F' episome and generated 4 x 10^{5}
25   colonies from a single solubilized precipitate.
Additionally, DNA restriction analysis was
carried out to determine the presence of heavy and
light chain inserts. DNA restriction analysis of the
clones revealed the presence of a Xho I and Xba I
30   fragment of 1.4 kb as expected for Fd-cpVIII fusion
construct and kappa chain insert.
These results give additional evidence for
antigen specificity and multivalency. In addition to
providing immunological parameters, this precipitation
35   offers possibilities for facile enrichment of antigen

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specific phage particles. In principle, phage containing specific antibodies can be highly enriched by precipitation with antigens (which may be cell surface markers, viral, bacterial as well as synthetic molecules). The washed antigen-antibody precipitates can be solubilized by the addition of excess antigen and viable phage recovered. For the recovery of rare species an immobilized antigen may be used which opens the way for differential affinity elution.

In order to demonstrate the utility of immobilized antigen for the enrichment of clones of defined binding specificity, a panning experiment was performed. An ampicillin resistant phagemid expressing an anti-tetanus Fab as a cpVIII fusion was constructed. Rescue of this clone with helper phage produced phage encoding the ampicillin resistant phagemid which displayed the anti-tetanus Fab on their coat. These phage encoding tetanus specificity were admixed with NPN hapten encoding phage (1:100) and allowed to bind to a microtitration plate coated with tetanus toxoid. Following a one hour maintenance period, the plate was washed extensively and phage were then eluted with a low pH buffer. Infection of XL1-Blue cells in log phase growth and subsequent plating of aliquots on ampicillin and chloramphenicol allowed for direct quantitation of enrichment. Examination of over 1,000 colonies showed that ampicillin resistant colonies derived from the eluted phage exceeded chloramphenicol resistant colonies by 27 to 1. Therefore, panning enriched the phage displaying the anti-tetanus Fab by 2700 fold. This result suggests that a clone of defined specificity present at one part per million will dominate over nonspecific clones following two rounds of panning.

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5.   Advantages of Assembling Combinatorial Antibody
      Fab Libraries Along Phage Surfaces

A powerful technique for generating libraries
with 10^{8-9} members and selecting from the library of
combinatorial Fabs with preselected binding
activities, , is presented. In the vector described
herein, the restriction cloning sites for inserting
PCR generated antibody fragments have been retained as
previously reported for the lambda vector. The rescue
of the genes encoding the antibody Fd and kappa chains
is mediated through the utilization of the f1 origin
of replication leading to the synthesis and packaging
of the positive strand of the vector on co-infection
with helper phage. Since the 'mature' virus particle
assembles by incorporating the major coat protein
around the single stranded DNA as it passes through
the inner membrane into the periplasmic space, not
only does it capture the genetic information carried
on the phagemid vector but also incorporates several
copies of functional Fab along the length of the
particle. On subsequent infection of hosts cells
carrying the F' episome the phagemid confers
resistance allowing selection of colonies on the
appropriate antibiotic. In essence, the antigen
recognition unit has been linked to instructions for
its production.

The full power of the earlier combinatorial
system could not be fully utilized since screening
allowed ready access to only about 0.1-1% of the
members. In the phagemid/M13 system similar size
libraries are generated and all the members are
accessed via affinity selection. Furthermore, unlike
the lambda vector which generated monovalent Fabs,
this system generates multivalent particles, thus
allowing the capture of a wider range of affinities.

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The unique phagemid restriction sites permit the recombination of Fd and kappa chains allowing chain replacement or shuffling. The rescue of filamentous single stranded DNA allows rapid sequencing and

5   analysis of the genetic make up of the clone of interest. Indeed it can be envisaged that phage encoding antibody specificity may be enriched by antigen selection prior to DNA sequencing or mutagenesis. The option to further develop an

10   iterative process of mutation followed by selection may allow the rapid generation of high affinity antibodies from germ line sequences. The process may be automated. Setting aside the potential of the system to mimic nature, the phagemid/M13 system would

15   allow a more complete dissection of the antibody response in humans which may yield useful therapeutic and diagnostic reagents.

The membrane anchoring of the heavy chain and the compartmentalization of the kappa chain in the

20   periplasm is the key to expressing this functional dimeric protein. The potential of this system is by no means limited to antibodies and may be extended to any protein recognition system or combination of systems containing multiple members. For example

25   coupling of ligand and effector systems in a high avidity matrix is now possible. In a similar vein a library of ligands can be sorted against a library of receptors.

30   6.   Randomized Mutagenesis of the CDR3 Region of a
          Heavy Chain Encoding Anti-Tetanus Toxoid
          a.   PCR Mutagenesis with Degenerate
               Oligonucleotides
          To obtain a mutagenized heterodimer of this

35   invention of altered specificity that would no longer

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recognize a tetanus toxoid antigen (TT) but would
recognize and specifically bind to a new antigen, a
method was developed to randomize only the CDR3 region
of a heavy chain fragment encoded by a known
nucleotide sequence. This approach is schematically
diagrammed in Figure 12 where a representative heavy
chain fragment within a phagemid clone, consisting of
alternating framework regions (1 through 4) shown by
white blocks and complementarity determining regions
(CDR) (1 through 3) shown by cross-hatched blocks and
the first constant region (CH1), is subjected to two
separate rounds of PCR. In the first PCR
amplification reaction, the 5' end of the heavy chain
beginning at framework 1 and extending to the 3' end
of framework 3 is amplified. In the second PCR
amplification reaction, the CDR3 region is randomly
mutagenized shown by the black box. This is
accomplished through the use of a pool of
oligonucleotide primers synthesized with a degenerate
region sandwiched between and contiguous with
conserved framework region 3 and 4 sequences. The
resulting amplification products from the second
amplification, each having a randomized CDR3 region,
have their 5' end at the 3' end of framework 3 and the
3' end of the product extends to the 3' end of the CH1
region.

The pool of degenerate oligonucleotide primers
have been designed to result in the amplification of
products having a 5' end that is complementary to and
will overlap with the 3' end of the products of the
first PCR reaction product. Thus, the two separate
PCR reaction products are pooled and subjected to a
third PCR reaction in which the overlapping region
between the two products is extended to result in
heavy chain having a randomized CDR3 region.

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A heavy chain DNA template for use in this
invention was available in a clone (a phagemid vector
designated 7E containing heavy and light chain
fragments) from a human combinatorial anti-tetanus
toxoid (TT) Fab library derived from lambda Hc2 and
Lc2 libraries as described by Persson et al., Proc.
Natl. Acad. Sci., USA, 88:2432-2436 (1991). To create
a semi-synthetic combinatorial library, clone 7E was
constructed in pComb2-3' dicistronic expression vector
for the expression of a heavy chain-cpIII membrane
anchor fusion protein (Fd-cpIII) and a soluble light
chain as described for anti-NPN in Example 2k by
Barbas et al., Proc. Natl. Acad. Sci., USA,
88:7978-7982 (1991).

The phagemid vector pComb2-3' was prepared as
described in Example 1b(ii). The original pairing of
the heavy and light chains from the 7E lambda clone
was maintained by ligation of the Xho I - Xba I
fragment into a Xho I - Xba I digested pComb2-3'
vector. To replace the cpIII membrane anchor
sequence, LacZ promoter sequence and pel B leader
deleted by the Xho I - Xba I digestion, a Spe I - Sac
I fragment from pComb3, as prepared in Example 1b(ii),
was ligated into the pComb2-3' vector containing the
heavy and light chain sequences from clone 7E. The
resultant phagemid clone, hereinafter referred to as
pC3-TT7E, was first expressed as described for
anti-NPN heterodimers on phage surfaces in Example 3
and subsequently screened by panning on TT-coated
plates as described for anti-NPN in Example 4c. Clone
pC3-TT7E exhibited a K_d towards TT on the order of 10^{-7}
M and was enriched over nonspecific phage by 10^3-fold
as described by Barbas et al., supra.

Clone pC3-TT7E, having both heavy and light chain
sequences, was used as the template DNA for the

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randomized mutagenesis of the CDR3 region of the heavy
chain to alter antigen binding specificity as
described herein. The sequence of the heavy chain was
determined as described in Example 1a(ii). Two
separate PCR reactions were performed as illustrated
in Figure 12.

The first PCR reaction resulted in the
amplification of the region of the heavy chain
fragment in the pC3-TT7E clone beginning of framework
region 1 and extending to the end of framework region
3 which is located 5' to CDR3 which is approximately
400 base pairs in length. To amplify this region, the
following primer pairs were used. The 5' anti-sense
oligonucleotide primer, FT3X, having the nucleotide
sequence 5'-G-CAA-TAA-ACC-CTC-ACT-AAA-GGG-3' (SEQ ID
NO 118), hybridized to the non-coding strand of the
heavy chain corresponding to the region 5' of and
including the beginning of framework 1. The 3' sense
oligonucleotide primer, B7EFR3, having the nucleotide
sequence 5'-TCT-CGC-ACA-ATA-ATA-CAC-GGC-3' (SEQ ID NO
119), hybridized to the coding strand of the heavy
chain corresponding to the 3' end of the framework 3
region. The oligonucleotide primers were synthesized
by Research Genetics (Hunstville, AL). The PCR
reaction was performed in a 100 μl reaction containing
one μg of each of oligonucleotide primers FTX3 and
B7EFR3, 8 μl 2.5 mM dNTP's (dATP, dCTP, dGTP, dTTP), 1
μl Taq polymerase, 10 ng of template pCE-TT7E, and
10μl of 10X PCR buffer purchased commercially (Promega
Biotech). Two drops of mineral oil were placed on top
of the admixture and 35 rounds of PCR amplification in
a thermocycler were performed. The amplification
cycle consisted of denaturing at 94°C for one minute,
annealing at 50°C for one minute, followed by
extension at 72°C for two minutes. The resultant PCR

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amplification products were then gel purified as
described in Example 1d and used in an overlap
extension PCR reaction with the products of the second
PCR reaction, both as described below, to recombine
5   the two products into reconstructed heavy chains
containing mutagenized CDR3 regions as illustrated in
Figure 12. The total yield of DNA from this
amplification was approximately 3μg/100μl.
The second PCR reaction resulted in the
10   amplification of the heavy chain from the 3' end of
framework region 3 extending to the end of CH1 region
which is approximately 390 base pairs in length. To
amplify this region, the following primers were used.
The 5' anti-sense oligonucleotide primer pool,
15   designated 7ECDR3, had the nucleotide sequence
represented by the formula,
5'-GTG-TAT-TAT-TGT-GCG-AGA-NNS-NNS-NNS-NNS-NNS-NNS-NNS
-NNS-NNS-NNS-NNS-NNS-NNS-NNS-NNS-NNS-TGG-GGC-CAA-GGG-A
CC-ACG-3' where N can be A, C, G, or T and S is either
20   C or G (SEQ ID NO 120), wherein the 5' end of the
primer pool was complementary to the 3' end of
framework 3 represented by the complementary
nucleotide sequence of the oligonucleotide primer
B73FR3 and the 3' end of the primer pool was
25   complementary to the 5' end of framework 4. The
region between the two specified ends of the primer
pool was represented by a 48-mer NNS degeneracy which
ultimately encoded a diverse population of mutagenized
CDR3 regions of 16 amino acid residues in length. The
30   3' sense oligonucleotide primer, CG1Z, as described by
Persson et al., supra, having the nucleotide sequence
5'-GCATGTACTAGTTTTGTCACAAGATTTGGG-3' (SEQ ID NO 121),
hybridized to the coding strand of the heavy chain
corresponding to the 3' end of the CH1. The second
35   PCR reaction was performed on the pC3-TT7E in a 100 μl

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reaction as described above containing one μg of each
of oligonucleotide primers 7ECDR3 and CG1Z. The
resultant PCR amplification products were then gel
purified as described above. The total yield of DNA

5   from this second PCR mutagenesis amplification was
approximately 3μg/100μl.

One hundred nanograms of gel purified products
from the first and second PCR reactions were then
admixed with 1 μg each of FTX3 and CG1Z

10   oligonucleotide primers as a primer pair in a final
PCR reaction to form a complete heavy chain fragment
by overlap extension as illustrated in Figure 12. The
PCR reaction admixture also contained 10 μl 10X PCR
buffer, 1 μl Taq polymerase and 8 ul 2.5 mM dNTP's as

15   described above. The PCR reaction was performed as
described above. To obtain sufficient quantities of
amplification product, 15 identical PCR reactions were
performed. The resulting heavy chain fragments
beginning at framework 1 and extending to the end of

20   CH1 and having randomly mutagenized CDR3 regions were
approximately 790 base pairs in length. The heavy
chain fragment amplification products from the 15
reactions were first pooled and then gel purified as
described above prior to their incorporation into a

25   phagemid library. The total yield of DNA from each
amplification was approximately 3μg/100μl thus the
total pooled yield contained approximately 45 μg of
amplified mutagenized heavy chain.

b.   Phagemid Library Construction

30   The resultant gel purified heavy chain
fragments prepared in Example 6a were then digested
with the restriction enzymes, Xho I and Spe I, as
described in Example 2d. The resultant digested heavy
chain fragments were subsequently gel purified prior

35   to insertion into the pC3-TT7E phagemid vector clone

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which was previously digested with the same
restriction enzymes to remove the non-mutagenized
heavy chain fragment and form a linear vector.
Ligation of 640 ng of the heavy chain Xho I Spe I
5   fragments having mutagenized CDR3 regions into two μg
of the linearized pC3-TT7E phagemid vector to form
circularized vectors having mutagenized CDR3 regions
was performed overnight at room temperature using 10
units of BRL ligase (Gaithersburg, MD) in BRL ligase·
10   buffer in a reaction volume of 150 μl. Five separate
ligation reactions were performed to increase the size
of the phage library having mutagenized CDR3 regions.
Thus, the total amount of amplified mutagenized heavy
chain for five ligation reactions was 3.2 μg.
15   Following the ligation reactions, the circularized DNA
was precipitated at -20°C for two hours by the
admixture of 2 μl of 20 mg/ml glycogen, 15 μl of 3 M
sodium acetate at pH 5.2 and 300 μl of ethanol. DNA
was then pelleted by microcentrifugation at 4°C for 15
20   minutes. The DNA pellet was washed with cold 70%
ethanol and dried under vacuum. The pellet was
resuspended in 10 μl of water and transformed by
electroporation into 300 μl of E. coli XL1-Blue cells
as described in Example 2k to form a phage library.
25   The total yield from the mutagenesis and
transformation procedure described herein was
approximately 5 X 10^7 transformants. XL1-Blue cells
from E. coli were selected as the hosts as the single
step codon TAG is suppressed.
30   After transformation, to isolate phage on which
heterodimer expression has been induced, for
subsequent panning on target antigens such as
fluorescein, 3 ml of SOC medium (SOC was prepared by
admixture of 20 g bacto-tryptone, 5 g yeast extract
35   and 0.5 g NaCl in one liter of water, adjusting the pH

to 7.5 and admixing 20 ml of glucose just before use
to induce the expression of the Fd-cpIII and light
chain heterodimer) was admixed and the culture was
shaken at 220 rpm for one hour at 37°C, after which
5   time 10 ml of SB (SB was prepared by admixing 30 g
tryptone, 20 g yeast extract, and 10 g Mops buffer per
liter with pH adjusted to 7) containing 20 μg/ml
carbenicillin and 10 μg/ml tetracycline and the
admixture was shaken at 300 rpm for an additional
10 hour. This resultant admixture was admixed to 100 ml
SB containing 50 μg/ml carbenicillin and 10 μg/ml
tetracycline and shaken for one hour, after which time
helper phage VCSM13 (10^{12} pfu) were admixed and the
admixture was shaken for an additional two hours.
15 After this time, 70 μg/ml kanamycin was admixed and
maintained at 30°C overnight. The lower temperature
resulted in better heterodimer incorporation on the
surface of the phage. The supernatant was cleared by
centrifugation (4000 rpm for 15 minutes in a JA10
20 rotor at 4°C). Phage were precipitated by admixture
of 4% (w/v) polyethylene glycol 8000 and 3% (w/v) NaCl
and maintained on ice for 30 minutes, followed by
centrifugation (9000 rpm for 20 minutes in a JA10
rotor at 4°C). Phage pellets were resuspended in 2 ml
25 of PBS and microcentrifuged for three minutes to
pellet debris, transferred to fresh tubes and stored
at -20°C for subsequent screening as described below.
    For determining the titering colony forming units
(cfu), phage (packaged phagemid) were diluted in SB
30 and 1 μl was used to infect 50 μl of fresh (OD600 = 1)
E. coli XLI-Blue cells grown in SB containing 10 ug/ml
tetracycline. Phage and cells were maintained at room
temperature for 15 minutes and then directly plated on
LB/carbenicillin plates.
35   c.   Selection of Anti-Fluorescein Heterodimers

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on Phage Surfaces

1)   Multiple Pannings of the Phage Library
      Having Mutagenized CDR3 Regions

The phage library produced in Example
6b having heavy chain fragments with mutagenized CDR3
regions was panned as described herein on a microtiter
plate coated with a 50 μg/ml fluorescein-BSA conjugate
to screen for anti-fluorescein heterodimers.
Fluorescein was conjugated to BSA according to the
methods described in "Antibodies: A Laboratory
Manual", eds Harlow et al., Cold Spring Harbor
Laboratory, 1988.

The panning procedure used was a modification of
that originally described by Parmley and Smith
(Parmley et al., Gene, 73:30-5-318). Two to four
wells of a microtiter plate (Costar 3690) were coated
overnight at 4°C with 25 μl of 50 μg/ml TT antigen
prepared above in 0.1 M bicarbonate, pH 8.6. The
wells were washed twice with water and blocked by
completely filling the well with 3% (w/v) BSA in PBS
and maintaining the plate at 37°C for one hour. After
the blocking solution was shaken out, 50 μl of the
phage library prepared above (typically 10^{11} cfu) were
admixed to each well, and the plate was maintained for
two hours at 37°C.

Phage were removed and the plate was washed once
with water. Each well was then washed ten times with
TBS/Tween (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5%
Tween 20) over a period of one hour at room
temperature where the washing consisted of pipetting
up and down to wash the well, each time allowing the
well to remain completely filled with TBS/Tween
between washings. The plate was washed once more with
distilled water and adherent phage were eluted by the
addition of 50 μl of elution buffer (0.1 M HCl,

adjusted to pH 2.2 with solid glycine, containing 1
mg/ml BSA) to each well followed by maintenance at
room temperature for 10 minutes. The elution buffer
was pipetted up and down several times, removed, and
5   neutralized with 3 μl of 2 M Tris base per 50 μl of
elution buffer used.

Eluted phage were used to infect 2 ml of fresh
(OD_{600} = 1) E. coli XL1-Blue cells for 15 minutes at
room temperature, after which time 10 ml of SB
10   containing 20 μg/ml carbenicillin and 10 μg/ml
tetracycline was admixed. Aliquots of 20, 10, and
1/10 μl were removed from the culture for plating to
determine the number of phage (packaged phagemids)
that were eluted from the plate. The culture was
15   shaken for one hour at 37°C, after which it was added
to 100 ml of SB containing 50 μg/ml carbenicillin and
10 μg/ml tetracycline and shaken for one hour. Helper
phage VCSM13 (10^{12} pfu) were then added and the
culture was shaken for an additional two hours. After
20   this time, 70 μg/ml kanamycin was added and the
culture was incubated at 37°C overnight. Phage
preparation and further panning were repeated as
described above.

Following each round of panning, the percentage
25   yield of phage were determined, where % yield =
(number of phage eluted/number of phage applied) X
100. The initial phage input ratio was determined by
titering on selective plates, as described in Example
6b, to be approximately 10^{11} cfu for each round of
30   panning. The final phage output ratio was determined
by infecting two ml of logarithmic phase XL1-Blue
cells as described above and plating aliquots on
selective plates.

As an alternative to elution with acid, phage
35   bound to the wells of the microtiter plate were eluted

by admixing 50 μl of a solution of 10^{-5} M fluorescein diluted in PBS followed by a maintenance period of one hour at 37°C. The solution was then pipetted up and down to wash the wells. The resultant eluate was

5   transferred to 2 ml of fresh E. coli XLI-Blue cells for infection as described above for preparing phage and further panning. In subsequent rounds of panning, phage were eluted with 10^{-6} M fluorescein.

The results of the amount of phage that were

10   specifically bound to fluorescein-coated wells over four consecutive rounds of panning and elution with acid or with fluorescein alone are shown below in Table 8. Comparable yields of phage on which heterodimers were expressed that bound specifically to

15   fluorescein were achieved with either elution protocol. These data confirm that mutagenesis of the CDR3 region as described in this invention resulted in the altering of a heterodimer which initially specifically bound to TT to one that specifically

20   bound fluorescein.

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Table 8

Phage Eluted

	Acid Elution	Fluorescein Elution
round 1	5.6×10^5/well	4.7×10^5/well
round 2	4.6×10^6/well	5.6×10^5/well
round 3	3.8×10^5/well	1.4×10^6/well
round 4	1.3×10^6/well	4.0×10^6/well

Nonspecific binding to this surface with control
phase varied between 10^4 and 10^5 phage per well.
Production of soluble Fab and verification of binding
to fluorescein by ELISA as described in 2) below
revealed 8 reactive clones of 60 randomly selected
from the transformed colonies and 38 reactive clones
of 40 randomly selected from the transformed colonies
for the acid eluted and fluorescein eluted libraries,
respectively.

2)   Preparation of Soluble Heterodimers for
       Characterizing Binding Specificity to
       Fluorescein

In order to further characterize the
specificity of the mutagenized heterodimers expressed
on the surface of phage as described above, soluble
Fab heterodimers from both acid eluted and fluorescein
eluted phage were prepared and analyzed in ELISA
assays on fluorescein-coated plates, by competitive
ELISA with increasing concentrations of soluble
fluorescein-BSA and also by fluorescence quenching
assays.   The latter assays were performed as described
in "Fluorescein Hapten: An Immunological Probe", ed
E. W. Voss, CRC Press, Inc. pp 52-54, 1984.

To prepare soluble heterodimers, phagemid DNA
from positive clones was isolated and digested with
Spe I and Nhe I.   Digestion with these enzymes
produced compatible cohesive ends.   The 4.7-kb DNA

fragment lacking the gene III portion was gel-purified
(0.6% agarose) and self-ligated. Transformation of E.
coli XL1-Blue afforded the isolation of recombinants
lacking the cpIII fragment. Clones were examined for
removal of the cpIII fragment by Xho I - Xba I
digestion, which should yield an 1.6-kb fragment.
Clones were grown in 100 ml SB containing 50 μg/ml
carbenicillin and 20 mM $MgCl_2$ at 37°C until an OD_{600} of
0.2 was achieved. IPTG (1 mM) was added and the
culture grown overnight at 30°C (growth at 37°C
provides only a light reduction in heterodimer yield).
Cells were pelleted by centrifugation at 4000 rpm for
15 minutes in a JA10 rotor at 4°C. Cells were
resuspended in 4 ml PBS containing 34 μg/ml
phenylmethylsulfonyl fluoride (PMSF) and lysed by
sonication on ice (2-4 minutes at 50% duty). Debris
was pelleted by centrifugation at 14,000 rpm in a JA20
rotor at 4°C for 15 minutes. The supernatant was used
directly for ELISA analysis as described below and was
stored at -20°C. For the study of a large number of
clones, 10ml cultures provided sufficient heterodimer
for analysis. In this case, sonications were
performed in 2 ml of buffer.

The soluble heterodimers prepared above were
assayed by ELISA. For this assay, 1 μg/well of
fluorescein-BSA solution was admixed to individual
wells of a microtiter plate and maintained at 4°C
overnight to allow the protein solution to adhere to
the walls of the well. After the maintenance period,
the wells were washed one time with PBS and thereafter
maintained with a solution of 3% BSA to block
nonspecific sites on the wells. The plates were
maintained at 37°C for one hour after which time the
plates were inverted and shaken to remove the BSA
solution. Soluble heterodimers prepared above were

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then admixed to each well and maintained at 37°C for
one hour to form immunoreaction products. Following
the maintenance period, the wells were washed ten
times with PBS to remove unbound soluble antibody and
then maintained with a secondary goat anti-human FAB
conjugated to alkaline phosphatase diluted in PBS
containing 1% BSA. The wells were maintained at 37°C
for one hour after which the wells were washed ten
times with PBS followed by development with
p-nitrophenyl phosphate (PNPP).

Immunoreactive heterodimers as determined in the
above ELISA were then analyzed by competition ELISA to
determine the affinity of the mutagenized
heterodimers. The ELISA was performed as described
above with increasing concentrations of soluble
fluorescein-BSA ranging in concentration from 10^{-9} M
up to 10^{-5} M in concentration admixed in the presence
of the soluble heterodimers. Maximal inhibition of
binding was achieved at a concentration of 10^{-6} M free
antigen with a half-maximal inhibition obtained with
approximately 10^{-7} M free antigen. Antibodies
expressed from all clones had approximate dissociation
constants (Kd) in the range of 10^{-7} to 10^{-8} M for
fluorescein-BSA conjugates for both the fluorescein
and acid elutions. True Kd's were determined in
fluorescence quenching assays. Antibodies expressed
on phage from clones following fluorescein elution had
higher affinities for fluorescein, 10^{-7} M versus 10^{-6} M
for the acid eluted antibodies. The parent clone, 7E,
showed no quenching within detectable limits of the
assay suggesting an affinity for free fluorescein less
than 10^{-5} M. The affinities of the tightest binders
of antibodies to fluorescein (10^{-7} M) approached the
average Kd of the secondary response of immunized mice
for free fluorescein (10^{-7} M) as shown by Kranz et

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al., Mol. Immunol., 20:1313-1322 (1983).

Thus, the mutagenized heterodimers of this invention specifically recognized and bound to fluorescein. Additional experiments were performed to confirm that the mutagenized heterodimers no longer recognized the TT to which the nonmutagenized heterodimer originally bound. Fluorescence quenching assays were also performed to confirm the specificity of binding of the mutagenized heterodimers. Soluble heterodimers prepared from phage that were either eluted with acid or with fluorescein alone were equally effective at binding fluorescein by any of the aforementioned approaches. The invention of mutagenesis of the CDR3 region of the heavy chain of a heterodimer described herein thus resulted in the alteration of binding specificity from TT to fluorescein.

    d.   Sequence Analysis of Selected
            Anti-Fluorescein Heterodimers

The complete nucleotide sequence of the mutated heavy chain of a representative number of fluorescein-BSA binding clones was determined. No PCR induced mutations outside of the CDR3 region were observed. The predicted amino acid sequences of the heavy chain CDR3 region are shown in Figure 13 with the corresponding SEQ ID NO shown in parenthesis. Seven clones recovered from the acid elution regimen showed no consensus sequence. The lack of consensus behavior in the acid eluted clones may be contributed by their recognition of a more complex epitope consisting of fluorescein and BSA and is reflected in the more disparate affinities to fluorescein and fluorescein-BSA.

Conversely, the clones isolated by fluorescein elution showed a high selection of consensus

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sequences. Of ten clones sequenced, only three
different sequences were observed. All of the clones
had a glycine residue at amino acid residue position
95 and an aspartic acid residue at position 101. The
5   amino acid residue positions are based on the Kabat
numbering system as described by Kabat et al., in
"Sequences of Proteins of Immunological Interest, U.S.
Department of Health and Human Services, (1987). Both
possible codons for encoding the glycine residue
10   provided by the synthesis protocol in which all 32
possible codons were produced were used. In natural
antibodies, the aspartic acid residue at position 101
usually plays a structural role by forming a salt
bridge with an arginine residue at position 94 in
15   framework region 3 (FR3). Thus, the artificial
selection process had recapitulated an interaction of
structural significance which mirrors that seen in the
animal.

In addition, nine of the semi-synthetic
20   antibodies expressed from ten clones contained a
serine-arginine-proline triplet sequence near the
center of the loop directly adjacent or one residue
removed on the amino-terminal side of an arginine
residue, though the codon usage for two of these
25   residues was different. Clone F31 lacked this central
motif. All sequences were rich in arginine residues
which was encoded by three of the 32 possible codons.
Comparison of the occurrence within the ten difference
CDR3 sequences of arginine with leucine and serine,
30   which were also encoded by three possible codons in
the synthesis, revealed a arginine-leucine-serine
ratio of 29:16:15. This bias towards selection of
arginine may be the result of the dianionic character
of fluorescein.
35   The finding that different codons were used

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support the proposition that clonal selection occurred
at the level of antigen-antibody union and not because
of some unexpected bias of nucleotide incorporation
into DNA.

5

7.   Randomized Mutagenesis of the CDR3 Region of a
       Light Chain Encoding Anti-Tetanus Toxoid
       a.   PCR Mutagenesis with Degenerate
              Oligonucleotides

10   Following a similar procedure to the random
mutagenesis of the heavy chain as described in Example
6, the CDR3 region of a light chain fragment of the
anti-tetanus toxoid specific phagemid clone pC3-TT7E
was randomized to produce antibodies having a

15   specificity for fluorescein. The PCR amplifications
were performed as described in Example 6a with the
exception of the oligonucleotides primers used in the
reactions.
              The first PCR reaction resulted in the

20   amplification of the region of the light chain
fragment in the pC3-TT7E clone from the 5' EcoR V site
of the vector extending into the 5' end of the CDR3
region. To amplify this region, the following primers
were used: The 5' anti-sense oligonucleotide primer,

25   KEF, having the nucleotide sequence
5'-GAATTCTAAACTAGCTAGTCG-3' (SEQ ID NO 126),
hybridized to the non-coding strand of the light chain
corresponding to the EcoR V site in the vector; The
3' sense oligonucleotide primer, KV12B, having the

30   nucleotide sequence 5'-ATACTGCTGACAGTAATACAC-3' (SEQ
ID NO 127), hybridized to the coding strand of the
heavy chain corresponding to the 5' end of CDR3. The
PCR amplification was performed as described in
Example 6a. The resultant PCR products were then gel

35   purified as described in Example 1d and used in an

overlap extension PCR reaction with the products of
the second PCR reaction, both as described below, to
recombine the two products into reconstructed heavy
chains containing mutagenized CDR3 regions as
illustrated in Figure 12.

The second PCR reaction resulted in the
amplification of the light chain from the 5' end of
the CDR3 region extending to the end of the CH1
region. To amplify this region, the following primers
were used. The 5' anti-sense oligonucleotide primer
pool, designated KV5R, had the nucleotide sequence
represented by the formula,
5'-TATTACTGTCAGCAGTATNNKNNKNNKNNKNNKACTTTCGGCGGAGGGAC
C-3' (SEQ ID NO 128) where N can be A, C, G or T and
where K is either G or T, wherein the 5' end of the
primer pool was complementary to the 5' end of CDR3
and the 3' end of the primer pool was complementary to
the 3' end of CDR3 and the 5' end of framework 4. The
region between the two specified ends of the primer
pool was represented by a 15-mer degeneracy which
ultimately encoded a diverse population of internal
mutagenized CDR3 regions of 5 amino acids in length
bordered by non-mutagenized 5' and 3' ends of the CDR3
regions. The 3' sense oligonucleotide primer, T7B,
having the nucleotide sequence
5'-AATACGACTCACTATAGGGCG-3" (SEQ ID NO 129),
hybridized to the coding strand of the light chain
corresponding to the T7 region in the vector. The
second PCR reaction was performed on the pC3-TT7E as
described in Example 6a with the KV5R and T7B primers.
The resultant PCR amplification products were then gel
purified as described above.

Five hundred nanograms of gel purified products
from the first and second PCR reactions were then
admixed with 1 μg each of KEF and T7B oligonucleotide

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primers as a primer pair in a final PCR reaction to
form a complete light chain fragment by overlap
extension as illustrated in Figure 12. The PCR
amplification was performed as described in Example
5   6a. To obtain sufficient quantities of amplification
product, 5 identical PCR reactions were performed.
The resulting light chain fragments, that began at the
5' EcoR V site and extended to the T7 region, had five
internal amino acids randomly mutagenized CDR3.
10   b.   Phagemid Library Construction
The resultant gel purified light chain
fragments prepared in Example 7a were then digested
with the restriction enzymes, Sac I and Xba I, as
described in Example 2d. The resultant light chain
15   fragments were subsequently gel purified prior to
ligation into the pC3-TT7E phagemid vector clone which
was previously digested with the same restriction
enzymes to remove the non-mutagenized light chain
fragment and form a linearized vector. Ligation of
20   450 ng of light chain amplification products into 1.4
µg linearized pC3-TT7E phagemid vector to form
circularized vectors having mutagenized CDR3 regions
was performed as described in Example 6b. Five
separate ligation reactions were performed to increase
25   the size of the phage library having internally
mutagenized CDR3 regions. Following the ligation
reactions, the circularized DNA was precipitated and
transformed into E. coli XLI-Blue as described in
Example 6b to form a phage library. The total yield
30   from the mutagenesis and transformation procedure
described herein was approximately 2 X 10⁷
transformants. Phage were isolated as described for
the heavy chain mutagenesis transformants.
c.   Selection of Anti-Fluorescein Heterodimers
35   on Phage Surfaces and Sequence Analysis of

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<u>Selected Antibodies</u>

The phage library produced in Example 7b having light chain fragments with five internally mutagenized amino acids in the CDR3 region was panned as described in Example 6c. The numbers of phage that were specifically bound to fluorescein-coated wells over three consecutive rounds of panning and hapten elution with a phage input of 10^{11} were 0.75 X 10^6, 1 X 10^6 and 2.4 X 10^7. The repeated cycles of transformation, phage preparation, panning and elution thus resulted in a significant enrichment of heterodimers that specifically bound to fluorescein. Soluble Fabs were prepared as described in Example 6b2) for characterizing binding specificity to fluorescein. Seven clones were selected for sequence analysis as described in Example 6d. The results of the sequence analysis are shown in Figure 14. The mutated region of the light chain CDR3 spans Kabat amino acid immunoglobulin light chain positions from 92 to 96. The sequence of this region from the starting clone, pC3-TT7E, was Gly-Ser-Ser-Leu-Trp (SEQ ID NO 148). Of the seven antibodies mutated and selected on fluorescein, five of them from clones P2, P21, P23, P28 and P19 had the amino acid sequence Thr-Arg-Pro-Gly-Val (SEQ ID NO 149) but each were the result of translations from unique nucleotide sequences. The two remaining antibodies from P15 and P11 clones, derived from unique nucleotide sequences, also had unique amino acid sequences. Thus, since most of the mutagenized light chains had the same amino acid sequence encoded by the possible codons in the synthesis, the artificial selection process has recapitulated an interaction of structural significance which in the animal resulted from the process of natural selection.

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Mutagenesis of the CDR regions is not limited to
the CDR3 region as primers can be designed to result
in the random mutagenesis of CDR1 and CDR2 of both the
heavy and the light chain. Mutating all six CDR
5   regions would result in an exceptionally diverse
library beyond that which can be obtained in the
animal. To obtain randomization in all the CDRs, the
heavy chain CDRs could first be randomized from a
starting clone followed by selection of the best
10   antibody binders. A second mutagenesis step on the
CDRs of the light chain could be performed and the
resultant library can be mixed with the selected heavy
chain binders. Alternatively, all CDRs could be
simultaneously randomized resulting in heavy and light
15   chain libraries which are then combined and subjected
to selection against a preselected antigen.
Thus, the Examples 6 and 7 illustrate a method
according to the present invention for mutagenizing
the heavy and light complementarity determining
20   regions (CDR) of an immunoglobulin gene, and also
illustrates oligonucleotides useful therefor.

8.   In Vitro Selection and Affinity Maturation of
Antibodies from a Naive Combinatorial
25   Immunoglobulin Library
A combinatorial immunoglobulin library approach
has been used to obtain monoclonal antibodies from
non-immune adult mice, thereby establishing the
principles of (i) accessing naive combinatorial
30   antibody libraries for predetermined specificities and
(ii) increasing the affinity of the selected antibody
binding sites by random mutagenesis. A combinatorial
Fab library expressing Igμ and k light chain fragments
on the surface of filamentous phage was prepared from
35   bone marrow of non-immunized, adult Balb/c mice with

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the multivalent display vector pComb8 prepared in
Example 1b(i). Phage displaying low affinity Fab's
having binding constants of approximately 10^4 to 10^5
M^{-1} specific for progesterone were isolated from the
library by their ability to bind the hapten. Random
mutagenesis of the heavy and light chain variable
regions expressed in the monovalent phage display
vector pComb3 was performed by error-prone PCR.
Clones with improved affinity for progesterone were
subsequently selected. Thus, as described herein,
antibodies with desirable characteristics from a non-
immune source were selected and affinity maturation
achieved by using the twin vectors pComb8 and pComb3,
thus opening the route to obtaining specific
antibodies from a generic library and bypassing
immunization.

The invention described herein has three
essential features: (i) the ability to initially
access low affinity Fabs from a naive library by the
use of multivalent phage expression systems, (ii)
subsequent affinity maturation by error-prone PCR and
(iii) the use of a single chain construct during the
maturation process to avoid a high background of
artifactual binding due to loss of the light chain.
When used in concert, these methods allowed for the
selection and affinity maturation of antibodies from a
naive library.

a.   RNA Isolation and cDNA Synthesis

Three non-immunized adult male (6 months)
Balb/cByJ mice (Scripps breeding colony) were used to
prepare 5×10^7 bone marrow cells in 4% fetal calf
serum in PBS. To deplete for surface IgG positive
cells, the preparation was maintained with rat-anti
mouse IgG_{2b} (0.1 ml), goat anti-mouse IgG (0.1 ml),
and rabbit anti-mouse IgG_{2b} (0.1 ml) for 30 minutes at

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ambient temperature. The cells were pelleted, washed
with PBS and resuspended in 9 ml PBS. Rabbit
complement was added (1 ml) and maintained at 37°C for
30 minutes. The cells were pelleted and total RNA
5   isolated as described in Example 2b. The total RNA
was used as a template for the cDNA synthesis for μ-
and k-chains with the following primers: Igμ, 5'-
ATTGGGACTAGTTTCTGCGACAGCTGGAAT-3' (SEQ ID NO 151) (the
Spe I restriction site sequence is underlined) and k,
10   5'-GCGCCGTCTAGAATTAACACTCATTCCTGTTGAA-3' (SEQ ID NO
152) (the Xba I restriction site is underlined)
respectively, using SuperScript Kit (BRL).

         Briefly, 7 μg of total RNA was admixed with 60
pmol of primer, heated to 70°C for 10 minutes and
15   immediately cooled on ice. Two μl of RNase inhibitor,
10 μl of 5x synthesis buffer, 8 μl of dNTP mix (to
give final concentration of 200 μM of each NTP), 5 μl
of 0.1 M DTT, and 1 μl of BRL SuperScript RT (200
U/μl) were admixed, and the reaction was made up to 50
20   μl with DEPC treated water. The reaction was allowed
to proceed at room temperature for 10 minutes and then
at 42°C for 50 minutes. The reaction was terminated
by maintaining at 90°C for 5 minutes and then placing
on ice for 10 minutes followed by admixing 1 μl of
25   RNase H and maintaining at 37°C for 20 minutes. PCR
amplification was performed in a 100 μl reaction
mixture as described in Example 2, using V_H 1-9 and
the μ chain primer for the heavy chains and V_L3-7 and
the k chain primers for the light chains as shown in
30   Table 5.

         b.   Naive Immunoglobulin μ/k Library
               Construction
               The PCR amplified μ-chain and k-chain DNA
were cleaved with Xho I-Spe I and Sac I-Xba I,
35   respectively. The resulting μ chain Xho I-Spe I

fragments were inserted into the pComb8 phagemid
vector prepared in Example 1b(i) to generate a μ
chain-cp VIII fusion library. Transformation into E.
coli XL1-Blue and phage production was carried out
essentially as described in Example 6. Subsequently,
the k light chain Sac I-Xba I fragments were cloned
into the heavy chain F_d μ-cpVIII fusion library.

A combinatorial library of 5×10^6 members was
established by subsequently cloning the Igμ F_d and k
light chain fragments into the pComb8 vector which
allowed the fusion of the heavy chain F_d fragment to
cpVIII. Since the Fab antibody fragments were
displayed at a high copy number on the phage surface,
this vector was selected for accessing low affinity
antibodies which are expected to be found in an
unselected and unprimed repertoire.

c.   Selection of Low Affinity Antibodies
      Specific for Progesterone

The recombinant phagemids prepared above
were packaged into M13 phage particles and five rounds
of panning on progesterone-3-(O-Carboxymethyl)-oxime-
BSA coated ELISA wells was performed as described in
Example 6. Briefly, wells of a microtitration plate
were coated at 4°C with 50 μl of 100 μg/ml
progesterone-3-(O-Carboxymethyl)-oxime BSA conjugate
(Sigma #P4778) in PBS. The wells were washed twice
with water and blocked by completely filling with 1%
w/v BSA in PBS and maintaining the plates at 37°C for
one hour. Blocking solution was flicked out and 50 μl
of the phage library (typically 10^{11} cfu) in PBS-BSA
(0.1% w/v) were admixed to each well and the plates
maintained for two hours at 37°C. The washing steps,
elution, and multiplication of the phage were done
essentially as described in Example 6a1).

Phage eluted after the first and third round were

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analyzed for expression of anti-progesterone Fab's by
bacterial colony lifts as described in Example 2f.
The colonies were probed for progesterone binding with
a progesterone-3-(O-Carboxymethyl)-oxime HRP
5   conjugate. The filters were developed using 4-
chloronaphthol. Care was taken to exclude artifacts
caused by phagemids expressing the Igμ F$_d$ cpVIII
fusion without a corresponding light chain. These
heavy chain-only phages reacted nonspecifically to
10   unrelated antigens such as BSA, HRP, hen egg lysozyme,
presumably due to the hydrophobic patch displayed on
an unpaired heavy chain.

Those colonies producing the strongest signal in
the western blot were further examined, and three
15   clones, PgA11, PgB6, and PgF1, were isolated for
subsequent analysis. The first two emerged from the
first round of panning, and the latter was isolated
after the third round of selection. All three Fab's,
produced in their soluble form as described in Example
20   6c2) bound specifically to progesterone-3-(O-
Carboxymethyl)-oxime-BSA and progesterone-11α-
hemisuccinyl-BSA. Additionally, all three Fab's
displayed a significant crossreactivity against an
epitope on cytochrome C. Their apparent binding
25   constants for progesterone-3-(O-carboxymethyl)-oxime-
BSA were determined as 10^4 M^{-1} for PgA11, and 3 x 10^4
M^{-1} and 10^5 M^{-1} for PgF1 and PgB6, respectively. These
binding constants were much below that reported for
anti-progesterone monoclonal antibodies with
30   affinities of 2 to 5 x 10^8 M^{-1}. Clones PgB6 and PgF1
utilized the same combination of closely related V$_H$
and V$_L$ genes. Both of their V$_H$ genes were identical
to two closely related but distinct germline genes
with no evidence of somatic mutation, as one would
35   expect for a naive repertoire. The true germline

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gene(s) for their closely related V_L genes are not yet
known. Since both the V_H genes have joined to
different D and J segments, the clones PgB6 and PgF1
cannot be of the same origin, but must have been
5   selected from two independent cloning events, pointing
towards a possible importance of this particular
combination for progesterone binding. The V_L and V_H
genes used by PgA11 are not closely related to the
other two clones.

10   Thus, this demonstrated that by using a
multivalent display vector, Fab's can be isolated from
naive combinatorial libraries with affinities
comparable to those observed for the primary immune
response to haptens, such as phosphorylcholine and
15   nitrophenol. Further, the combinatorial library
approach can yield V genes or V gene combinations
which would not have been selected in vivo.

         d.   Affinity Maturation by PCR Directed
                Mutagenesis
20   To mimic the process of somatic mutation
which leads to the selection of antibodies with a
higher affinity, random mutations were created in both
the V_L and V_H regions and antibodies were subsequently
selected with an increased affinity to the hapten
25   progesterone. In order to target the mutations by
error-prone PCR specifically and only to the V
regions, a single chain fusion plasmid Fv-cpIII was
constructed in a pComb2-3 phagemid vector which
contained in frame fusions of the following elements:
30   the pelB leader sequence for secretion, the V_H and V_L
reading frames linked with a synthetic oligonucleotide
coding for a flexible peptide as discussed in Example
6a, and the cpIII moiety. The use of a single chain
vector further overcomes the difficulties due to
35   unwanted selection for nonspecific binding by phage

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which express Ig heavy chains only.

For preparing a single-chain heavy and light
chain (designated F$_v$) fusion with the membrane anchor
cpIII, the plasmid pComb2-3 prepared in Example 1b(ii)
5   having only one Spe I restriction site, was digested
with the restriction endonucleases Xba I and Nhe I and
religated, thus eliminating the light chain cloning
cassette. A synthetic DNA linker which encodes a 15
amino acid linker sequence consisting of two
10   oligonucleotides, a 5' anti-sense primer having the
nucleotide sequence 5'-
TCGAGAAAGTCTCTAGAGGTAAATCTTCTGGTTCTGGTTCCGAATCTAAATCTA
CTGAGCTCAAAGTCA-3' (SEQ ID NO 153) and a 3' sense
primer having the nucleotide sequence 5'-
15   CTAGTGACTTTGAGCTCAGTAGATTTAGATTCGGAACCAGAACCAGAAGATTTA
CCTCTAGAGACTTTC-3 (SEQ ID NO 154) was inserted into
the Xho I-Spe I digested, truncated pComb2-3 vector
forming the phagemid ScpComb2-3. The internal
recognition sequence for restriction endonucleases Xba
20   I (TCTAGA) and Sac I (GAGCTC) are underlined. The V$_H$
and V$_L$ segments of the progesterone binders were then
amplified by PCR as described in Example 2g in two
separate reactions.

In the first PCR amplification, the primer
25   corresponding to SEQ ID NO 153 listed above and the
oligonucleotide having the nucleotide sequence 5'-
ATTTGGGAAGGACTGTCTAGATGMRGAGAC-3', (SEQ ID NO 155),
where M is either A or C and R is either A or G, were
used to amplify the heavy chain fragment. The light
30   chain fragment was separately amplified with the
primer corresponding SEQ ID NO 154 listed above and
the oligonucleotide having the nucleotide sequence 5'-
GAGGACTAGTTACAGTTGGTGCAGCATCAG-3' (SEQ ID NO 156. The
internal recognition sequences for Xba I (TCTAGA) and
35   Spe I (ACTAGT) are underlined. The V$_H$ and V$_L$ PCR

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fragments were digested with Xho I-Xba I and Sac I-Spe
I, respectively, and subsequently inserted into Scp-
Comb2-3.

      e.   Expression and Detection of Soluble Fabs and
5   Single Chain Fusion Antibodies Specific for
                    Progesterone

For Fab production, the gene VIII moiety in
the phagemids encoding the progesterone binders PgA11,
PgB6, and PgF1 was excised with restriction
10   endonucleases Spe I and EcoR I and subsequently
replaced by a synthetic linker encoding a TAA stop
codon (underlined). The linker was formed by the
oligonucleotides 5'-CTAGTTAACTGAGTAAG-3' (SEQ ID NO
157) and 5'AATTCTTACTCAGTTAA-3' (SEQ ID NO 158). The
15   production and detection of antibody Fab fragment was
performed essentially as described in Example 6c2),
except that the $E.$ $coli$ cells were disrupted by three
freeze thaw cycles. For producing soluble antibody
fragments, the V_H-linker-V_L fusions were excised from
20   the ScpComb2-3 phagemid with Xho I and Spe I and
subcloned into the expression vector pTAC01
(Pharmacia) which is a derivative of pF1260. The
pTAC01 vector has the inducible tac promoter, the pelB
leader sequence for secretion and allowed for in-frame
25   fusion of the inserted single chain fusion construct
with a decapeptide sequence as described in Example 1a
as a tag for immunochemical detection. Expression and
detection of the single chain fusion antibody
fragments was as described above, except that an anti-
30   decapeptide antibody conjugated to alkaline
phosphatase was used for the ELISA.

Three single chain fusion clones were selected by
the screening protocol and designated ScpComb2-3-PgF1,
-PgB6 and -PgA11. These resultant plasmids were
35   subjected to error-prone PCR mutagenesis as described

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below.

    f.   <u>Targeted Mutagenesis of the Heavy and Light</u>
<u>Chains by Error-Prone PCR</u>

        Equal amounts of undigested PgF1, PgB6, and
PgA11 ScpComb2-3 plasmids prepared above were admixed
and serially diluted. Aliquots of 100 ng, 10 ng, 1
ng, 0.1 ng, and 0.01 ng of the admixtures were
subjected separately to 35 cycles (1 minute at 94°C, 2
minutes at 50°C, 1 minute at 72°C) of amplification
under the following reaction conditions: 50 mM KCl, 10
mM Tris-Hcl (pH 9.0), 6.5 mM $MgCl_2$, 0.5 mM $MnCl_2$,
0.01% gelatin, 0.1% Triton X-100, 1 mM each dCTP,
dGTP, dTTP, 0.2 mM dATP, 0.1 mM dITP, using the M 13
reverse sequencing primer, 5'-AACAGCTATGACCATG-3' (SEQ
ID NO 159), and a backward primer complementary to the
cpIII moiety, 5'-GACAGGAGGTTGAGGCAGGT-3' (SEQ ID NO
160) at 100 μM. The basic method of error-prone PCR
was as originally described by Leung et al., <u>J.</u>
<u>Methods Cell. Mol. Biol.</u>, 1:11-15 (1989) which is
incorporated herein by reference. The DNA region to
be mutagenized was PCR amplified under conditions that
reduced the fidelity of DNA synthesis by Taq DNA
polymerase. As shown by Leung et al., <u>supra</u>, the
concentrations of the reagents $MuCl_2$ and dATP used in
the PCR amplifications resulted in 1.0% and 1.4%
mutation frequency, respectively. Thus, the frequency
of mutation increased as the dATP concentration
decreased.

        The PCR reactions of all template dilutions were
pooled and treated with phenol prior to digestion with
Xho I and Spe I. The gel purified and digested PCR
fragments were ligated back into the Xho I-Spe I
digested ScpComb2-3 plasmid. The ligation products
were electroporated into <u>E. coli</u> XL1-Blue giving rise
to 10^6 transformants. Subsequent steps of phage

-183-

production and panning were carried out as described
in Example 6, except that the phage were panned in the
absence of BSA.

Thus, a library of mutated anti-progesterone
single chain phagemid antibodies was established and
panned on progesterone-3-(O-Carboxymethyl)-oxime-BSA
and the number of eluted cfu was taken as a measure
for the relative affinity of the displayed single
chain antibodies to the hapten. After the third round
of panning a 50 to 100-fold increase in the yield of
eluted phagemids relative to the non-mutated
population was noted. Individual mutants showed a 10
to 300-fold increase in yield after panning as
compared to the parent clones, indicating that the
mutants encoded antibody binding sites with an
increased affinity. The four best mutants, designated
ScPgB6-1, -2, -3 and -4, were chosen for determination
of their affinity for the hapten-conjugate and
sequence analysis.

    g.   Determination of Affinity of Mutagenized
          Single Chain Fusion Anti-Progesterone
          Antibodies

The binding constants of the soluble
antibody fragments prepared form the four best
mutants, ScPgB6-1, -2, -3 and -4 chosen above were
determined by competitive ELISA as described in
Example 6c2). Briefly, wells of a microtitration
plate were coated at 4°C with 50 μl of 100 μg/ml
progesterone-3-(O-Carboxymethyl)-oxime-BSA conjugate
in PBS. The wells were washed twice with water and
blocked with 1% w/v BSA in PBS at 37°C for one hour.
Fab or single chain fusion supernatants were mixed
with progesterone-3-(O-Carboxymethyl)-oxime-BSA in
PBS-BSA (0.1% w/v), and maintained in the wells at
37°C for two hours. The plates were washed with PBS-

-184-

Tween (0.05% v/v), and goat anti-mouse k-chain
alkaline phosphatase conjugate (Southern Biotech) or
mouse anti-decapeptide monoclonal antibodies
conjugated to alkaline phosphatase was admixed and
5   maintained for one hour at 37°C. The plates were
washed as before and substrate was admixed (0.1 ml, p-
nitrophenyl phosphate at 1 mg/ml in 0.1 M Tris, pH 9.4
containing 50 mM MgCl$_2$). After maintenance at 25°C
for 60-180 minutes, the absorbance was read at 405 nm.
10   Apparent affinities were determined as the reciprocal
of the hapten concentration required to inhibit 50% of
the maximal binding in a competitive ELISA. This was
a close approximation to the affinity and permitted
the ranking of the binding activities.
15   The affinity of the mutated Sc antibodies to
progesterone-3-(O-carboxymethyl)-oxime-BSA as
determined by competitive ELISA had increased over the
parent ScPgB6 antibody by 30-fold for ScPgB6-1, and
approximately 13-fold for both ScPgB6-3 and ScPgB6-4.
20   Interestingly, the clone with the least mutations
exhibited the highest affinity. The crossreactivity
pattern for the mutant Sc antibodies did not change,
except that ScPgB6-1 had lost most of its reactivity
to cytochrome C. In extensively studies immune
25   responses to haptens, an increase in affinity by one
order of magnitude could be assigned to specific
single amino acid substitutions, which implies that
only one or two amino acid exchanges in the combining
site of the Sc anti-progesterone antibodies may have
30   accounted for their increased affinity to the hapten-
conjugate. Since a mutant with only a single amino
acid exchange was not recovered, the critical
residue(s) could not be identified that caused the
enhanced affinity to the hapten-conjugate. Further,
35   amino acid substitutions common to all three of the

mutants were not observed which suggested that
changing different residues can account for the
increased affinity to 3-(O-carboxymethyl)-
progesterone. A Ser_{64} to Pro_{64} substitution in the V_H
5   CDR2 is common to the mutants ScPgB6-3 and ScPgB6-4.
Although both mutants showed a similar affinity to the
hapten-conjugate, assessing the importance of that
residue for antigen binding cannot unequivocally be
done, since multiple amino acid exchanges occurred in
10   the V_L and V_H regions of the two mutants.

  h.   Nucleic Acid Sequencing

            The complete nucleotide sequences of the
variable regions of the heavy and light chains were
determined from double stranded DNA using Sequenase
15   2.0 (United States Biochemical). DNA sequencing
revealed that all four mutant clones, ScPgB6-1, -2, -3
and -4, had arisen from ScPgB6, with ScPgB6-1 and
ScPgB6-2 being identical. The predominant type of
mutation obtained by this PCR protocol was an A-G/T-C
20   nucleotide exchange (68%), while T-G/A-C, G-A/C-T, T-
A/A-T, G-C/C-G, or C-A/G-T exchanges occurred at
approximately the same frequency. DNA sequences with
a higher than average mutation frequency, i.e.
mutational hot spots, were observed. In addition, the
25   three mutant clones differed in the number of basepair
changes. The mutation frequencies for both the V_H and
V_L regions were found to be 1.5% for ScPgB6-1, 2.1%
for ScPgB6-3, and 4.1% for ScPgB6-4, which led to
multiple amino acid substitutions in the CDR's and
30   framework regions of the mutants.

            In summary, this invention contemplates the
principle of selection and affinity maturation of
specific antibodies to a hapten from a naive library.
Although in vitro selection and mutagenesis system is
35   quite simple compared to the complexity of the immune

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system, some common features exist: (i) The affinity
of antibodies selected from a naive combinatorial
library can reach the same order of magnitude as
antibodies from a primary immune response to haptens;
5   (ii) Although the mechanisms generating mutations _in
vivo or _in vitro_ were different, mutational ·hot spots
were observed; (iii) The increase in affinity after
one round of mutation and selection _in vitro_ is in the
same order of magnitude as observed for the transition
10   from primary to secondary immune responses to haptens;
and (iv) A mutated antibody combining site with an
altered crossreactivity was recovered as is
occasionally observed _in vivo_.
      When the combinatorial antibody approach was
15   first described, whether it could be used to
efficiently tap into the vast antibody repertoire _in
vivo was questionable. The present invention shows
that antibody chain combinations can be accessed and
evolved which may never be selected _in vivo_. Thus, it
20   now seems as if it is possible to exceed the diversity
of the antibody response _in vivo_ by molecular cloning
techniques.

      The foregoing is intended as illustrative of the
25   present invention but not limiting. Numerous
variations and modifications can be effected without
departing from the true spirit and scope of the
invention.

187
SEQ ID NO 17

5'

Ala Glu Gly Asp Asp Pro Ala Lys Ala Ala Phe Asn Ser Leu

Gln Ala Ser Ala Thr Glu Tyr Ile Gly Tyr Ala Trp Ala Met

Val Val Val Ile Val Gly Ala Thr Ile Gly Ile Lys Leu Phe

Lys Lys Phe Thr Ser Lys Ala Ser

188
SEQ ID NO 99

5'

GGCCGCAAATTCTATTTCAAGGAGACAGTCATAATGAAATACCTATTGCCTACG

GCAGCCGCTGGATTGTTATTACTCGCTGCCCAACCAGCCATGGCCCAGGTGAAA

CTGCTCGAGTCAGGACCTGGCCTCGTGAAACCTTCTCAGTCTCTGTCTCTCACC

TGCTCTGTCACTGACTACTCCATCACCAGTGCTTATTACTGGAACTGGATCCGG

CAGTTTCCAGGAAACAAACTGGAATGGATGGGCTACATAAGCTACGACGGTGTC

AATAAGTATGATCCATCTCTCAAGAATCGAATCTCCATCACTCGTGACACATCT

AACAATCAGTTTTTCCAGAAGTTGATTTCTGTGACTTCTGAGGACACAGGAACA

TATGACTGTTCAAGAGGGACTAGGGCCTCTGCTATGGACTACTGGGGTCAAGGA

ATTTCAGTCACCGTCTCCTCAGCCAAAACGACACCCCCATCTGTCTATCCACTG

GCCCCTGGATCTGCTGCCCAAACTAACTCCATGGTGACCCTGGGATGCCTGGTC

AAGGGCTATTTCCCTGAGCCAGTGACAGTGACCTGGAACTCTGGATCCCTGTCC

AGCGGTGTGCACACCTTCCCAGCTGTCCTGCAGTCTGACCTCTACACTCTGAGC

AGCTCAGTGACTGTCCCCTCCAGCCCTCGGCCCAGCGAGACCGTCACCTGCAAC

GTTGCCCACCCGGCCAGCAGCACCAAGGTGGACAAGAAAATTGTGCCCAGGGAT

TGTACTAGTTACCCGTACGACGTTCCGGACTACGGTTCTTAA

189

SEQ ID NO 100

5'

TGAATTCTAAACTAGTCGCCAAGGAGACAGTCATAATGAAATACCTATTGCCTACG

GCAGCCGCTGGATTGTTACTCGCTGCCCAACCAGCCATGGCCGAGCTCCAGATGAC

CCAGTCTCCAGCCTCCCTATCTGCATCTGTGGGAGAAACTGTCACCATCACATGTC

GATCAAGTGAGAATATTACAATTACT

190
SEQ ID NO 101

5'

CTGATGCTGCACCAACTGTATCCATCTTCCCACCATCCAGTGAGCAGTTAACATCT

GGAGGTGCCTCAGTCGTGTGCTTCTTGAACAACTTCTACCCCAAAGACTACAATGT

CAAGGGGAAGATTGATGGCAGTGAACGACAAAATGGCGTCCTGAACAGTTGGACTG

ATCAGGACAGCAAAGACAGCACCTACAGCATGAGCAGCACCCTCACGTTGACCAAG

GACGAGTATGAACGACATAACAGCTATACCTGTGATGCCACTCACAAGACATCAAC

TTCACCCATTGTCAAGAGCTTCAACAGGAATGAGTGTTAATTCTAGACGGCGC

191
SEQ ID NO 102

5'

GCTGAGGGTGACGATCCCGCAAAAGCGGCCTTTAACTCCCTGCAAGCCTCAGCGAC

CGAATATATCGGTTATGCGTGGGCGATGGTTGTTGTCATTGTCGGCGCAACTATCG

GTATCAAGCTGTTTAAGAAATTCACCTCGAAAGCAAGC

192

SEQ ID NO 111

5'

GTGCCCAGGGATTGTACTAGTGCTGAGGGTGACGATCCCGCAAAAGCGGCCTTTAA

CTCCCTGCAAGCCTCAGCGACCGAATATATCGGTTATGCGTGGGCGATGGTTGTTG

TCATTGTCGGCGCAACTATCGGTATCAAGCTGTTTAAGAAATTCACCTCGAAAGCA

AGCTGATAGAATTCGAGT

193
SEQ   ID   NO   112

5'

CCATTCGTTTGTGAATATCAAGGCCAAGGCCAATCGTCTGACCTGCCTCAA

CCTCCTGTCAATGCTGGCGGCGGCTCTGGTGGTGGTTCTGGTGGCGGCTCT

GAGGGTGGTGGCTCTGAGGGTGGCGGTTCTGAGGGTGGCGGCTCTGAGGGA

GGCGGTTCCGGTGGTGGCTCTGGTTCCGGTGATTTTGATTATGAAAGATG

GCAAACGCTAATAAGGGGGCTATGACCGAAAATGCCGATGAAAACGCGCTA

CAGTCTGACGCTAAAGGCAAACTTGATTCTGTCGCTACTGATTACGGTGCT

GCTATCGATGGTTTCATTGGTGACGTTTCCGGCCTTGCTAATGGTAATGGT

GCTACTGGTGATTTTGCTGGCTCTAATTCCCAAATGGCTCAAGTCGGTGAC

GGTGATAATTCACCTTTAATGAATAATTTCCGTCAATATTTACCTTCCCTC

CCTCAATCGGTTGAATGTCGCCCTTTTGTCTTTAGCGCTGGTAAACCATAT

GAATTTTCTATTGATTGTGACAAAATAAACTTATTCGGTGTCTTTGCGTTT

CTTTTATATGTTGCCACCTTTATGTATGTATTTTCTACGTTTGCTAACATA

CTGCGTAATAAGGAGTCTTAATCATGCCAGTTCTTTTGGGTATTCCGTTAT

TAT

SEQ ID NO 113

5'

GAGACGACTAGTGGTGGCGGTGGCTCTCCATTCGTTTGTGAATATCAAGGC

CAAGGCCAATCGTCTGACCTGCCTCAACCTCCTGTCAATGCTGGCGGCGGC

TCTGGTGGTGGTTCTGGTGGCGGCTCTGAGGGTGGTGGCTCTGAGGGTGGC

GGTTCTGAGGGTGGCGGCTCTGAGGGAGGCGGTTCCGGTGGTGGCTCTGGT

TCCGGTGATTTTGATTATGAAAAGATGGCAAACGCTAATAAGGGGGCTATG

ACCGAAAATGCCGATGAAAACGCGCTACAGTCTGACGCTAAAGGCAAACTT

GATTCTGTCGCTACTGATTACGGTGCTGCTATCGATGGTTTCATTGGTGAC

GTTTCCGGCCTTGCTAATGGTAATGGTGCTACTGGTGATTTTGCTGGCTCT

AATTCCCAAATGGCTCAAGTCGGTGACGGTGATAATTCACCTTTAATGAAT

AATTTCCGTCAATATTTACCTTCCCTCCCTCAATCGGTTGAATGTCGCCCT

TTTGTCTTTAGCGCTGGTAAACCATATGAATTTTCTATTGATTGTGACAAA

ATAAACTTATTCCGTGGTGTCTTTGCGTTTCTTTTATATGTTGCCACCTTT

ATGTATGTATTTTCTACGTTTGCTAACATACTGCGTAATAAGGAGTCTTAA

TCATGCCAGTTCTTTTGGGTATTCCGTTATTATGCTAGCTAGTAA

195

SEQ   ID   NO   114

5'

TATGCTAGCTAGTAACACGACAGGTTTCCCGACTGG

AAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGT

TAGCTCACTCATTAGGCACCCCAGGCTTTACACTTT

ATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGC

GGATAACAATTTCACACAGGAAACAGCTATGACCAT

GATTACGAATTCGAGCTCGGT

196
SEQ ID NO 115

5'

AGGTCCAGCTTCTCGAGTCTGGACCTGGCCTCGTGAAACCTTCTCAGTCTCTGTCT

CTCACCTGCTCTGTCACTGACTACTCCATCACCAGTGCTTATTACTGGAACTGGAT

CCGGCAGTTTCCAGGAAACAAACTGGAATGGATGGGCTACATAAGCTACGACGGTG

TCAATAAGTATGATCCATCTCTCAAGAATCGAATCTCCATCACTCGTGACACATCT

AACAATCAGTTTTTCCAGAAGTTGATTTCTGTGACTTCTGAGGACACAGGAACATA

TGACTGTTCAAGAGGGACTAGGGCCTCTGCTATGGACTACTGGGGTCAAGGAATTT

CAGTCACCGTCTCCTCAGCCAAAACGACACCCCATCTGTCTATCCACTGGCCCCT

GGATCTGCTGCCCAAACTAACTCCATGGTGACCCTGGGATGCCTGGTCAAGGGCTA

TTTCCCTGAGCCAGTGACAGTGACCTGGAACTCTGGATCCCTGTCCAGCGGTGTGC

ACACCTTCCCAGCTGTCCTGCAGTCTGACCTCTACACTCTGAGCAGCTCAGTGACT

GTCCCCTCCAGCCCTCGGCCCAGCGAGACCGTCACCTGCAACGTTGCCCACCCGGC

CAGCAGCACCAAGGTGGACAAGAAAATTGTGCCCAGGGATTGTACTAGTGCTGAGG

GTGACGATCCCGCAAAAGCGGCCTTTAACTCCCTGCAAGCCTCAGCGACCGAATAT

ATCGGTTATGCGTGGGCGATGGTTGTTGTCATTGTCGGCGCAACTATCGGTATCAA

GCTGTTTAAGAAATTCACCTCGAAAGCAAGCTGATAGAATTCGAGT

197

SEQ ID NO 116

5'

ATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTTATTACTCGCTGCCC

AACCAGCCATGGCCCAGGTGAAACTGCTCGAGATTTCTAGACTAGTGCTGAG

GGTGACGATCCCGCAAAAGCGGCCTTTAACTCCCTGCAAGCCTCAGCGACCG

AATATATCGGTTATGCGTGGGCGATGGTTGTTGTCATTGTCGGCGCAACTAT

CGGTATCAAGCTGTTTAAGAAATTCACCTCGAAAGCAAGCTGATAGAATTCG

SEQ ID NO 117

5'

GTACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCG

CAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTTGCTTT

CTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAA

TCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCC

CAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATA

GACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACT .

CTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGA

TTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGAT

TTAACAAAAATTTAACGCGAATTTTAACAAAATATTAACGTTTACAATTTA

AA

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What Is Claimed Is:

1.   A filamentous phage encapsulating a genome encoding a ligand-binding heterodimeric receptor.

2.   The filamentous phage of claim 1 wherein said receptor is an epitope-binding complex.

3.   The filamentous phage of claim 1 wherein said phage is detectably labeled.

4.   A ligand-binding heterodimeric receptor comprising first and second polypeptides, said first polypeptide flanked by an amino-terminal prokaryotic secretion signal domain and a carboxy-terminal filamentous phage membrane anchor domain, and said second polypeptide fused to an amino-terminal prokaryotic secretion signal domain.

5.   The receptor of claim 4 wherein said receptor is an epitope-binding complex.

6.   The receptor of claim 5 wherein said first polypeptide is an antibody heavy chain polypeptide and said second polypeptide is an antibody light chain polypeptide.

7.   The receptor of claim 5 wherein said first polypeptide is an antibody light chain polypeptide and said second polypeptide is an antibody heavy chain polypeptide.

8.   The receptor of claim 4 wherein said prokaryotic secretion signal is a pelB secretion signal.

9.   The receptor of claim 8 wherein said pelB secretion signal has an amino acid residue sequence represented by a formula selected from the group consisting of:

          (a)   SEQ ID NO 5,
          (b)   SEQ ID NO 6, and
          (c)   SEQ ID NO 7.

10.   The receptor of claim 4 wherein said

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filamentous phage membrane anchor is the cpVIII
membrane anchor.

    11.   The receptor of claim 10 wherein said
filamentous phage membrane anchor has an amino acid
5   residue sequence represented by the formula in SEQ ID
NO. 17 from residue 26 to residue 40.

    12.   The receptor of claim 4 wherein said
filamentous phage membrane anchor is the cpIII
membrane anchor.

10   13.   The receptor of claim 11 wherein said
filamentous phage membrane anchor has an amino acid
residue sequence represented by the formula in SEQ ID
NO. 16 from residues 1 to 211.

    14.   A vector for expressing a fusion
15   polypeptide, said vector comprising upstream and
downstream translatable DNA sequences operatively
linked via a sequence of nucleotides adapted for
directional ligation of an insert DNA, said upstream
sequence encoding a prokaryotic secretion signal, said
20   downstream sequence encoding a filamentous phage
membrane anchor, said translatable DNA sequences
operatively linked to a set of DNA expression signals
for expression of said translatable DNA sequences as
portions of said fusion polypeptide.

25   15.   The vector of claim 14 wherein said
prokaryotic secretion signal is a pelB secretion
signal.

    16.   The vector of claim 15 wherein said pelB
secretion signal has an amino acid residue sequence
30   represented by a formula selected from the group
consisting of:

         (a)   SEQ ID NO 5,
         (b)   SEQ ID NO 6, and
         (c)   SEQ ID NO 7.
35   17.   The vector of claim 14 wherein said

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filamentous phage membrane anchor is the cpVIII
membrane anchor.

18.   The vector of claim 17 wherein said
filamentous phage membrane anchor has an amino acid
residue sequence represented by the formula in SEQ ID
NO. 17 from residue 26 to residue 40.

19.   The vector of claim 14 wherein said
filamentous phage membrane anchor is the cpIII
membrane anchor.

20.   The vector of claim 19 wherein said
filamentous phage membrane anchor has an amino acid
residue sequence represented by the formula in SEQ ID
NO. 16 from residues 1 to 211.

21.   The vector of claim 14 further comprising a
filamentous phage origin of replication.

22.   The vector of claim 14 wherein said set of
expression signals includes a promoter, a ribosome
binding site, and at least one stop codon in frame
with said downstream translatable DNA sequence.

23.   The vector of claim 14 wherein said vector
has a nucleotide sequence shown in SEQ. ID. NO. 116
from base 1 to base 208.

24.   The vector of claim 14, further comprising a
second upstream translatable DNA sequence encoding a
prokaryotic secretion signal operatively linked via a
sequence of nucleotides adapted for directional
ligation of a second insert DNA, said second
translatable DNA sequence operatively linked to a set
of DNA expression signals for expression of said
second translatable DNA sequences as a portion of a
second fusion polypeptide.

25.   The vector of claim 24 wherein said
prokaryotic secretion signal has an amino acid residue
sequence represented by a formula selected from the
group consisting of:

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      (a)   SEQ ID NO 5,

      (b)   SEQ ID NO 6, and

      (c)   SEQ ID NO 7.

      26.   The vector of claim 24 wherein said vector has a nucleotide sequence shown in SEQ ID NO 3 from base 36 to base 118.

      27.   The vector of claim 24 wherein said DNA expression vector is the vector pCOMB8, pCOMB2-8 or pCKAB8.

      28.   The vector of claim 24 wherein said DNA expression vector is the vector pCOMB3, pCOMB2-3, pCOMB2-3' or pCKAB3.

      29.   A polypeptide comprising a ligand-binding receptor polypeptide operatively linked at the amino-terminus to a prokaryotic secretion signal domain and operatively linked at the carboxy-terminus to a filamentous phage membrane anchor domain.

      30.   The polypeptide of claim 29 wherein the polypeptide is an antibody variable chain polypeptide.

      31.   The polypeptide of claim 30 wherein the variable chain is an antibody heavy chain polypeptide.

      32.   A library of filamentous phage particles wherein each phage particle contains a DNA expression vector according to claim 14 or claim 24.

      33.   The library of claim 32 wherein said library contains at least 10^7 different species of said DNA expression vector.

      34.   A library of filamentous phage particles wherein each phage particle contains at least one ligand-binding heterodimeric receptor according to claim 4.

      35.   An oligonucleotide useful as a primer for inducing mutagenesis in a complementarity determining region (CDR) of an immunoglobulin gene, said oligonucleotide having 3' and 5' termini and

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comprising:

      a)   a nucleotide sequence at said 3'
terminus capable of hybridizing to a first framework
region of an immunoglobulin gene;

      b)   a nucleotide sequence at said 5'
terminus capable of hybridizing to a second framework
region of an immunoglobulin gene; and

      c)   a nucleotide sequence between said 3'
and 5' termini according to the formula:

$$[NNR]_n,$$

wherein N is independently any nucleotide, R is S, K
or analogs thereof, and n is 3 to about 24, wherein S
is G or C, K is G or T, said 3' and 5' terminal
nucleotide sequences having a length of about 6 to 50
nucleotides, and sequences complementary thereto.

    36.   The oligonucleotide of claim 35 wherein said
3' terminus has the nucleotide sequence 5'-
TGGGGCCAAGGGACCACG-3' (SEQ ID NO 122), and sequences
complementary thereto.

    37.   The oligonucleotide of claim 35 wherein said
5' terminus has the nucleotide sequence 5'-
GTGTATTATTGTGCGAGA-3' (SEQ ID NO 123) and sequences
complementary thereto.

    38.   The oligonucleotide of claim 35 wherein said
immunoglobulin is human.

    39.   The oligonucleotide of claim 35 wherein said
CDR is CDR3.

    40.   The oligonucleotide of claim 35 according to
the formula:
5'-GTGTATTATTGTGCGAGA[NNR]$_n$TGGGGCCAAGGGACCACG-3' (SEQ
ID NO 124).

    41.   The oligonucleotide of claim 40 wherein n is
16, and R is S (SEQ ID NO 120).

    42.   A method for inducing mutagenesis in a
complementarity determining region (CDR) of an

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immunoglobulin gene which comprises amplifying a CDR
portion of the immunoglobulin gene by polymerase chain
reaction (PCR) using a PCR primer oligonucleotide,
said oligonucleotide having 3' and 5' termini and
comprising:

    a)   a nucleotide sequence at said 3'
terminus capable of hybridizing to a first framework
region of an immunoglobulin gene;

    b)   a nucleotide sequence at said 5'
terminus capable of hybridizing to a second framework
region of an immunoglobulin gene; and

    c)   a nucleotide sequence between said 3'
and 5' termini according to the formula:

$$[NNR]_n,$$

wherein N is independently any nucleotide, R is S, K
or analogs thereof, and n is 3 to about 24, wherein S
is G or C, K is G or T, said 3' and 5' terminal
nucleotide sequences having a length of about 6 to 50
nucleotides, and sequences complementary thereto.

    43.   The method of claim 42 wherein said 3'
terminus has the nucleotide sequence 5'-
TGGGGCCAAGGGACCACG-3' (SEQ ID NO 122), and sequences
complementary thereto.

    44.   The method of claim 42 wherein said 5'
terminus has the nucleotide sequence 5'-
GTGTATTATTGTGCGAGA-3' (SEQ ID NO 123) and sequences
complementary thereto.

    45.   The method of claim 42 wherein said
immunoglobulin is human.

    46.   The method of claim 42 wherein said CDR is
CDR3.

    47.   The method of claim 42 according to the
formula:

5'-GTGTATTATTGTGCGAGA[NNR]$_n$TGGGGCCAAGGGACCACG-3' (SEQ
ID NO 124).

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48. The method of claim 47 wherein n is 16, and
R is S (SEQ ID NO 120).

49. A method of producing a library of DNA
molecules, each DNA molecule comprising a cistron for
expressing a polypeptide on the surface of a
filamentous phage particle, which method comprises:

        (a) forming a ligation admixture by
combining in a ligation buffer:

                (i) a repertoire of polypeptide genes
in the form of dsDNA, each vector having cohesive
termini adapted for directional ligation, and

                (ii) a plurality of DNA expression
vectors in linear form, each vector having upstream
and downstream cohesive termini (a) adapted for
directionally receiving said polypeptide genes in a
common reading frame, and (b) operatively linked to
respective upstream and downstream translatable DNA
sequences, said upstream translatable DNA sequence
encoding a prokaryotic secretion signal, said
downstream translatable DNA sequence encoding a
filamentous phage membrane anchor, and said
translatable DNA sequences operatively linked to
respective upstream and downstream DNA expression
control sequences; and

        (b) subjecting said admixture to ligation
conditions for a time period sufficient to operatively
link said polypeptide genes to said vectors, thereby
forming said library.

50. A method of producing a library of
dicistronic DNA molecules, each dicistronic DNA
molecule comprising first and second cistrons for
expressing first and second polypeptides of a
heterodimeric receptor on the surface of a filamentous
phage, which method comprises:

        (a) forming a first ligation admixture by

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combining in a ligation buffer:

(i)   a repertoire of first polypeptide
genes in the form of linear dsDNA, each having
cohesive termini adapted for directional ligation, and

5   (ii)   a plurality of DNA expression
vectors in linear form, each vector having upstream
and downstream first cohesive termini that are (a)
adapted for directionally receiving one of said first
polypeptide genes in a common reading frame, and (b)

10   operatively linked to respective upstream and
downstream translatable DNA sequences, said upstream
translatable DNA sequence encoding a prokaryotic
secretion signal, said downstream translatable DNA
sequence encoding a filamentous phage membrane anchor,

15   and said translatable DNA sequences operatively linked
to respective upstream and downstream DNA expression
control sequences; and

(b)   subjecting said admixture to ligation
conditions for a time period sufficient to operatively

20   link said first polypeptide genes to said vectors and
produce a plurality of circular DNA molecules each
having said first cistron for expressing said first
polypeptide;

(c)   producing a plurality of DNA

25   expression vectors in linear form, each linear vector
having second upstream and downstream cohesive termini
(i) adapted for directionally receiving one of a
repertoire of second polypeptide genes in a common
reading frame, and (ii) operatively linked to

30   respective upstream and downstream DNA sequences, said
upstream DNA sequence being a translatable sequence
encoding a prokaryotic secretion signal, said
downstream DNA sequence having at least one stop codon
in said reading frame, and said translatable DNA

35   sequence operatively linked to a DNA expression

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control sequence, by treating said plurality of
circular DNA molecules to restriction endonucleolytic
conditions sufficient to cleave said circular DNA
molecules and form said second cohesive termini;

5   (d)   forming a second ligation admixture by
combining in a ligation buffer:

                      (i)   said plurality of DNA expression
vectors formed in step (c), and

                      (ii) said repertoire of second
10   polypeptide genes in the form of dsDNA, each having
cohesive termini adapted for directional ligation to
said plurality of linear DNA vectors; and

                      (e)   subjecting said second admixture to
ligation conditions for a time period sufficient to
15   operatively link said second polypeptide genes to said
vectors and produce a plurality of circular DNA
molecules each having said second cistron for
expressing said second polypeptide, thereby forming
said library.

20   51.   The method of claim 50 wherein said
heterodimeric receptor is an epitope-binding complex.

            52.   A library of dicistronic DNA molecules each
comprising first and second cistrons of expressing
first and second polypeptides of a heterodimeric

25   receptor on the surface of a filamentous phage, said
library produced according to the method of claim 50.

            53.   A method for changing the diversity of a
library of filamentous phage particles comprising the
steps of:

30   a)   providing a library of filamentous
phage particles according to claim 32;

                b)   contacting the provided library with a
preselected ligand under conditions sufficient for
members of the library to bind to the ligand and form

35   a ligand-phage particle complex; and

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c)   isolating phage particles in said
complex away from non-bound library members to form a
ligand-enriched library comprising phage particles
having binding specificity for said preselected
ligand.

54.   The method of claim 53 wherein said
preselected ligand is affixed to a solid support, said
complex is in the solid phase and said isolating
comprises the steps of:

i)   washing the solid support to rinse
non-bound library members from the solid support; and

ii)   eluting solid-phase bound phage
particles to form said isolated phage particles.

55.   The method of claim 54 wherein said eluting
comprises contacting said solid-phase bound phage
particles with an elution buffer having a pH of from
pH 2 to pH 6.

56.   The method of claim 54 wherein said elution
comprises contacting said solid-phase bound phage
particles with an elution buffer containing said
preselected ligand.

57.   The method of claim 54 wherein said
filamentous phage membrane anchor is the cpVIII
membrane anchor.

58.   The method of claim 54 wherein said
filamentous phage membrane anchor is the cpIII
membrane anchor.

59.   A method for increasing the diversity of a
library of filamentous phage particles comprising the
steps of:

a)   providing a library of filamentous
phage particles according to claim 32;

b)   mutating the immunoglobulin variable
domain-coding nucleotide sequence present in each DNA
expression vector in the library to form a library of

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phage particles each containing a mutated
immunoglobulin variable domain nucleotide sequence.

60.   A method for maturing the affinity of an
epitope-binding complex encoded by a filamentous phage
according to claim 2 comprising the steps of:

a)   providing the genome of said
filamentous phage;

b)   mutating the immunoglobulin variable
domain-coding nucleotide sequence present in the
provided genome to form a library of phage particles
containing a mutated immunoglobin variable domain
nucleotide sequence;

c)   contacting the library formed in step
(b) with a preselected ligand under conditions
sufficient for members of the library to bind to the
ligand and form a ligand-phage particle complex; and

d)   isolating phage particles in said
complex away from non-bound library members to form a
ligand-enriched library comprising phage particles
having binding specificity for said preselected
ligand.

61.   The method of claim 60 wherein said mutating
comprises subjecting said immunoglobulin variable
domain-coding nucleotide sequence to an error-prone
polymerase chain reaction.

62.   The method of claim 60 wherein said mutating
comprises subjecting said immunoglobulin variable
domain-coding nucleotide sequence to the method of
claim 35 for mutating a CDR of the immunoglobulin
variable domain-coding nucleotide sequence.

63.   The method of claim 60 wherein said
immunoglobulin variable domain is V_H.

64.   A method for detecting the presence of a
preselected ligand in a sample comprising the steps
of:

-210-

a)   admixing a sample suspected to contain a preselected antigen with a ligand-binding heterodimeric receptor according to claim 4 that binds to said preselected ligand under binding conditions sufficient for said ligand-binding heterodimeric receptor to bind said ligand and form a ligand-receptor complex; and

b)   detecting the presence of said ligand-receptor complex or said ligand-binding heterodimeric receptor.

65.   The method of claim 64 wherein said ligand-binding heterodimeric receptor is on the surface of a filamentous phage particle.

66.   The method of claim 65 wherein said ligand-binding heterodimeric receptor is fused to a filamemtous phage membrane anchor selected from the group consisting of cpIII and cpVIII.

67.   The method of claim 65 wherein said detecting comprises detecting the presence of said filamentous phage particle, and thereby the presence of said product.

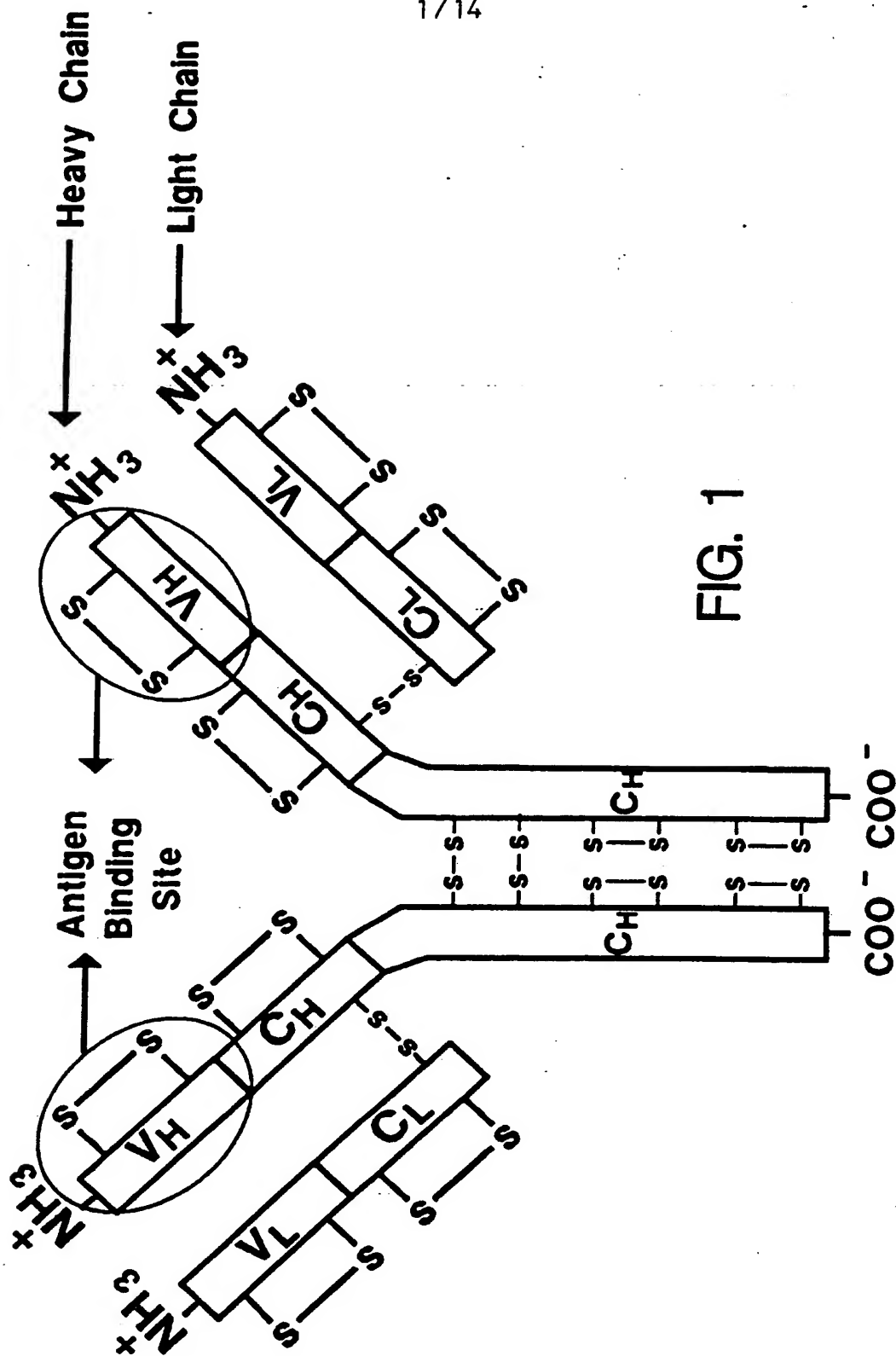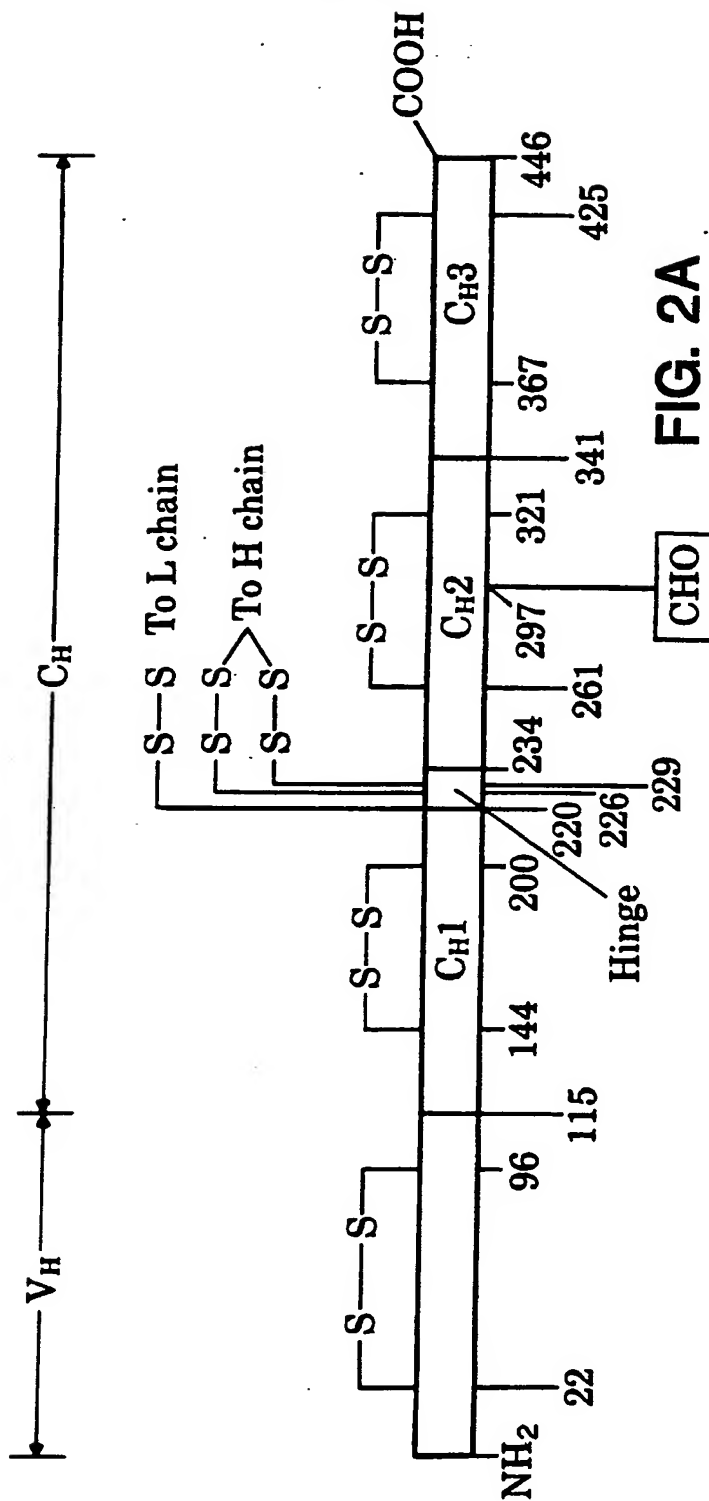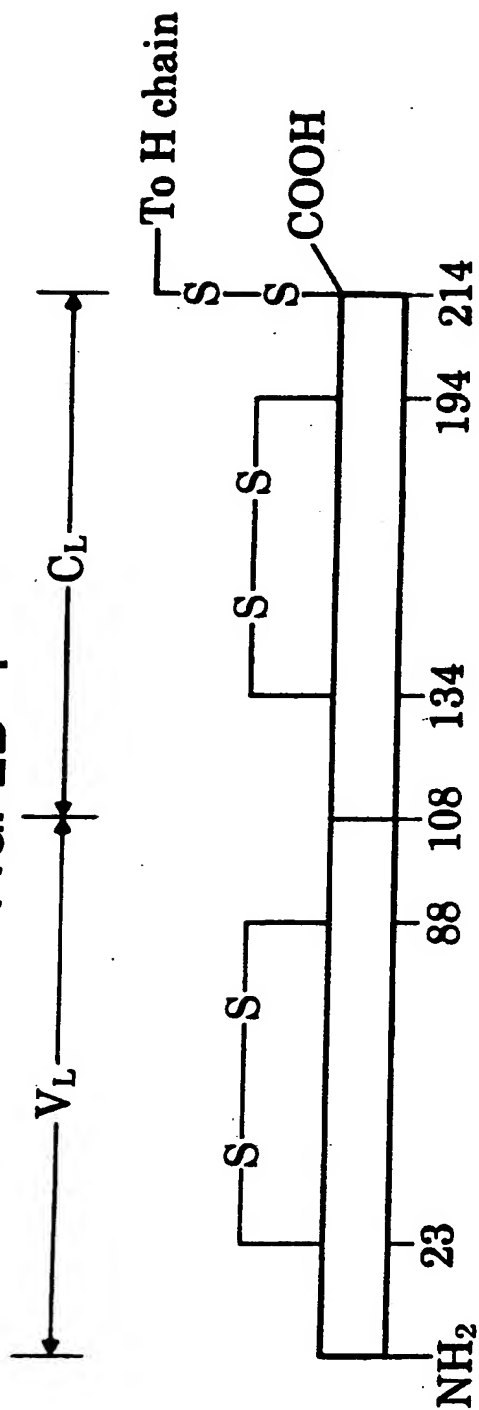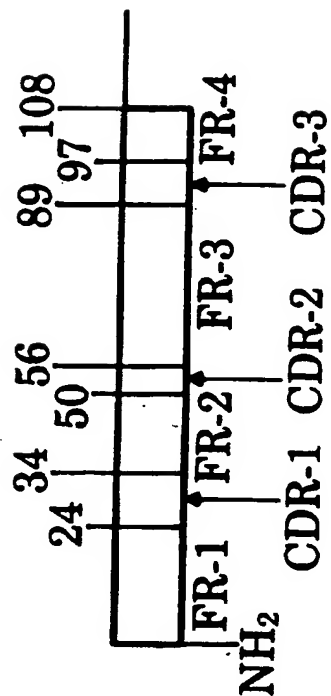FIG. 1

FIG. 2A

FIG. 2B-1

FIG. 2B-2

SHINE-DALGARNO MET

GGCCGCAAATTCTATTTCAAGGAGACAGTCATAATG
CGTTTAAGATAAAGTTCCTCTGTCAGTATTAC

LEADER SEQUENCE

AAATACCTATTGCCTACGGCAGCCGCT
TTTATGGATAACGGATGCCGTCGGCGA

LEADER SEQUENCE

GGATTGTTATTACTCGCTGCCCAACCAG
CCTAACAATAATGAGCGACGGGTTGGTC
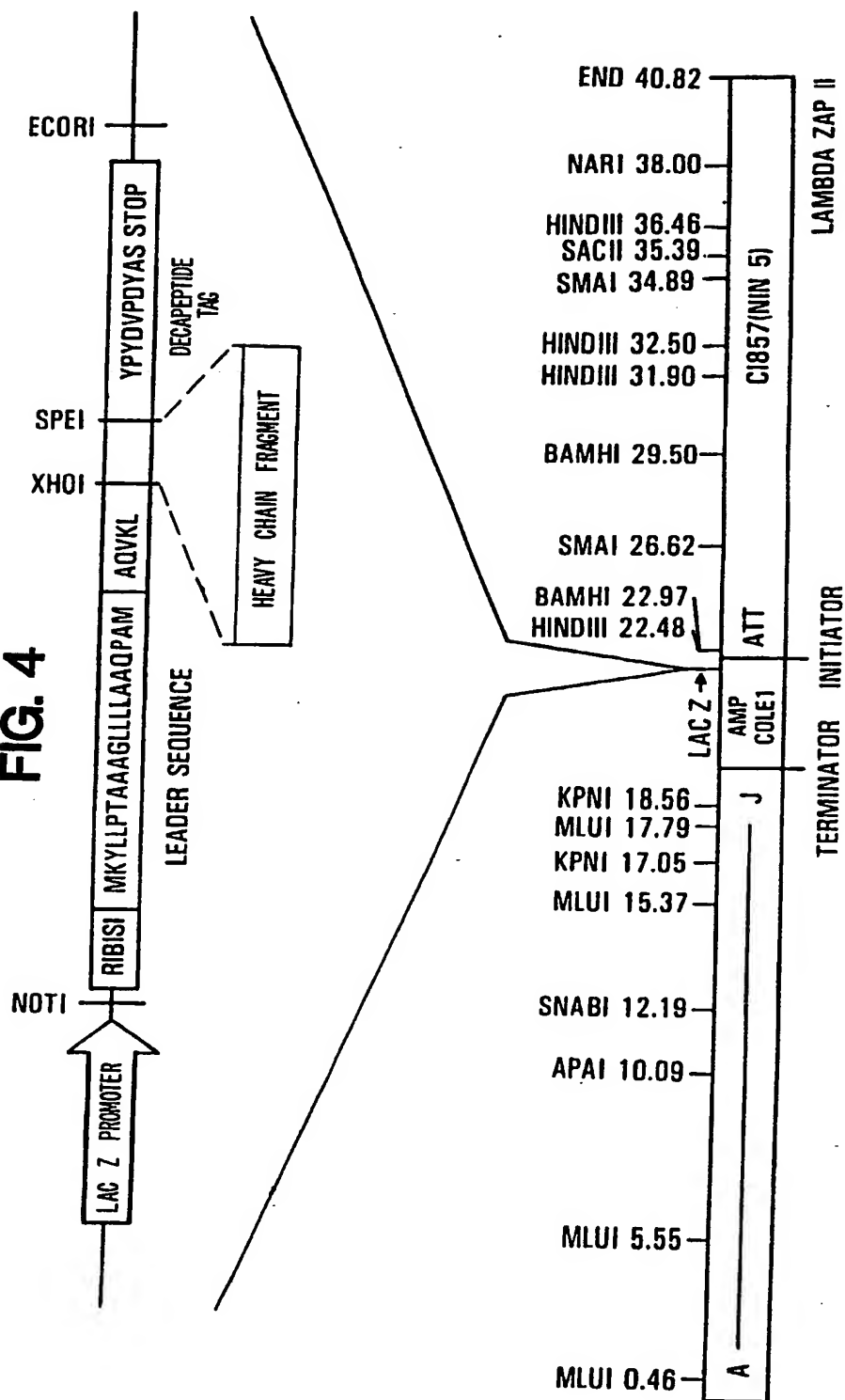
LINKER   LINKER

| NCOI | V_H | BACKBONE | XHOI | SPEI |

CCATGGCCCAGGTGAAACTGCTCGAGATTTCTAGACTAGT
GGTACCGGGTCCACTTTGACGAGCTCTAAAGATCTGATCA

STOP   LINKER

TyrProTyrAspValProAspTyrAlaSer
TACCCGTACGACGTTCCGGACTACGGTTCTTAATAGAATTCG
ATGGGCATGCTGCAAGGCCTGATGCCAAGAATTATCTTAAGCAGCT

FIG. 3

FIG. 4

LAC Z PROMOTER

NOTI

RIBISI | MKYLLPTAAAGLLLLAAQPAM | AQVKL

LEADER SEQUENCE

XHOI

SPEI

YPYDVPDYAS STOP

DECAPEPTIDE TAG

ECORI

HEAVY CHAIN FRAGMENT

LAMBDA ZAP II

END 40.82

NARI 38.00

HINDIII 36.46
SACII 35.39
SMAI 34.89

HINDIII 32.50
HINDIII 31.90

BAMHI 29.50

SMAI 26.62

BAMHI 22.97
HINDIII 22.48

CI857(NIN 5)

ATT

INITIATOR

AMP
COLE1

LAC Z

TERMINATOR

KPNI 18.56
MLUI 17.79
KPNI 17.05

MLUI 15.37

J

SNABI 12.19

APAI 10.09

MLUI 5.55

MLUI 0.46

A

FIG. 5

ECOR I　　　SHINE-DALGARNO　MET

TGAATTCTAAACTAGTCGCCAAGGAGACAGTCATAATGAAAT
TCGAACTTAAGATTTGATCAGCGGTTCCTCTGTCAGTATTACTTTA

LEADER SEQUENCE

ACCTATTGCCTACGGCGAGCCGCTGGATTGTTATTACTCGCTGCCCAACCAG
TGGATAACGGATGCCGTCGGCGACCTAACAATAATGAGCGACGGGTTGGTC

NCO I　　SAC I　　XBA I　　Not I

CCATGGCCGAGCTCGTCAGTTCTAGAGTTAAGCGGCCG
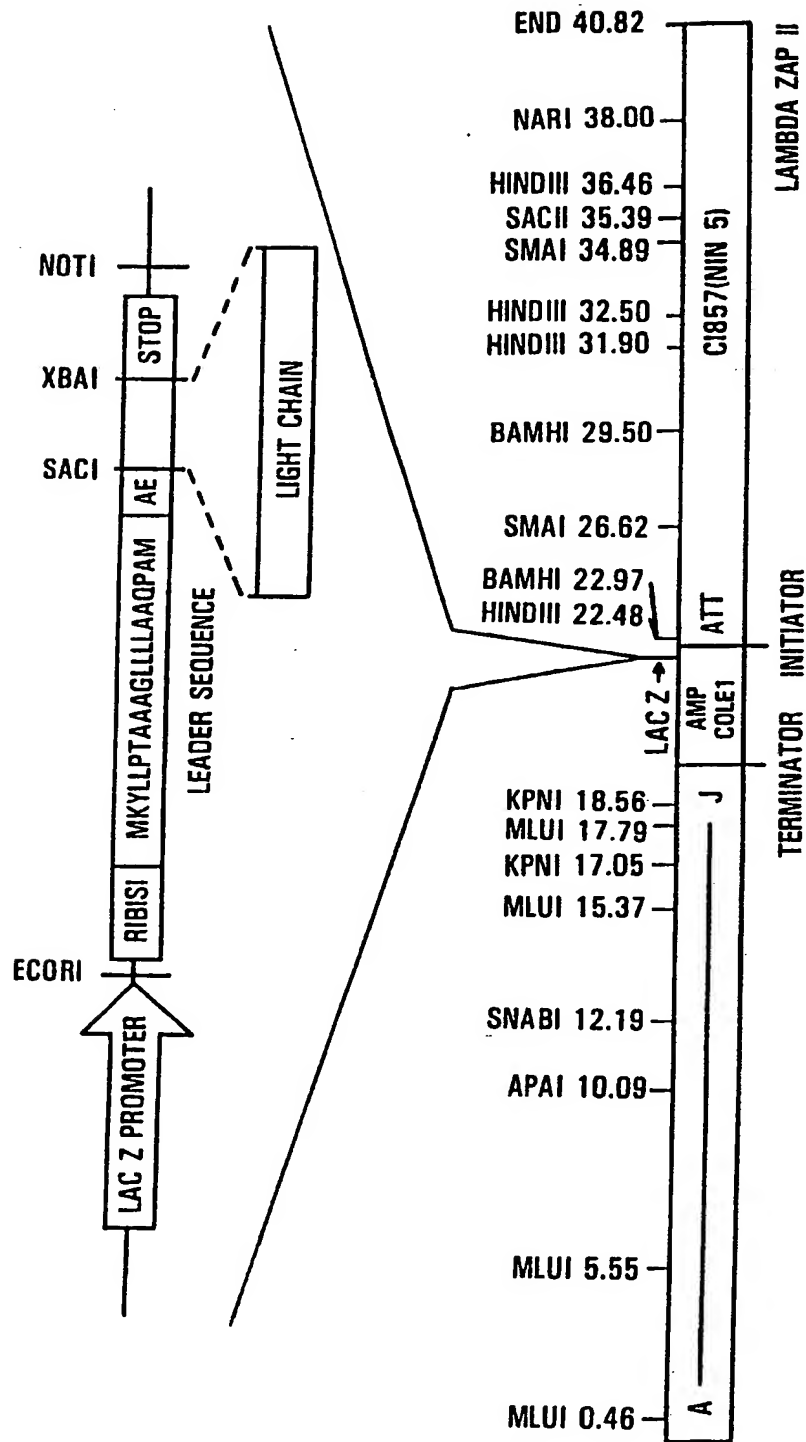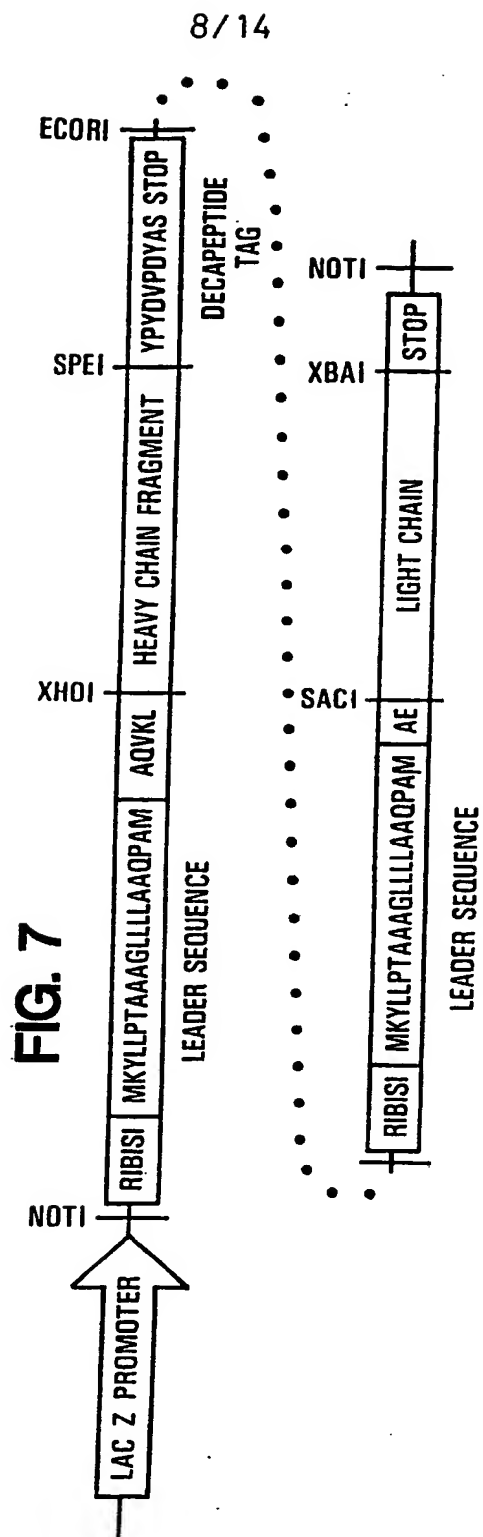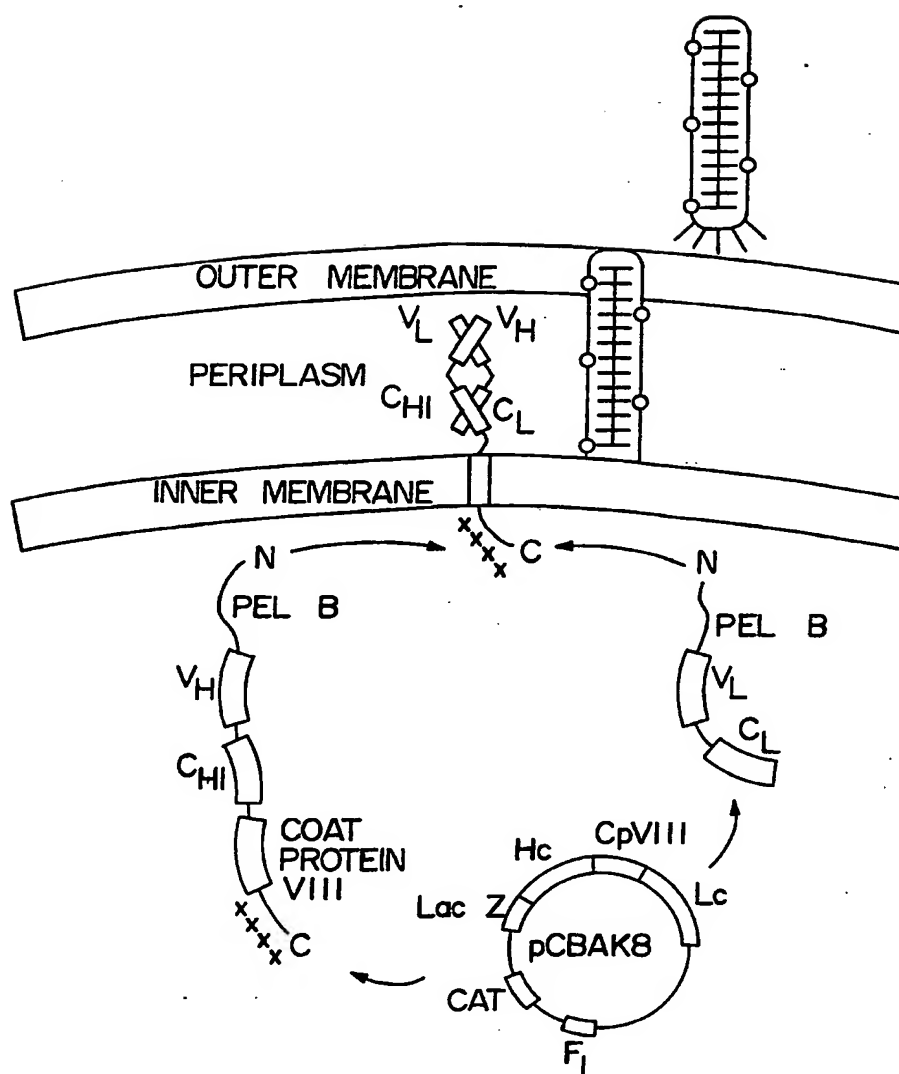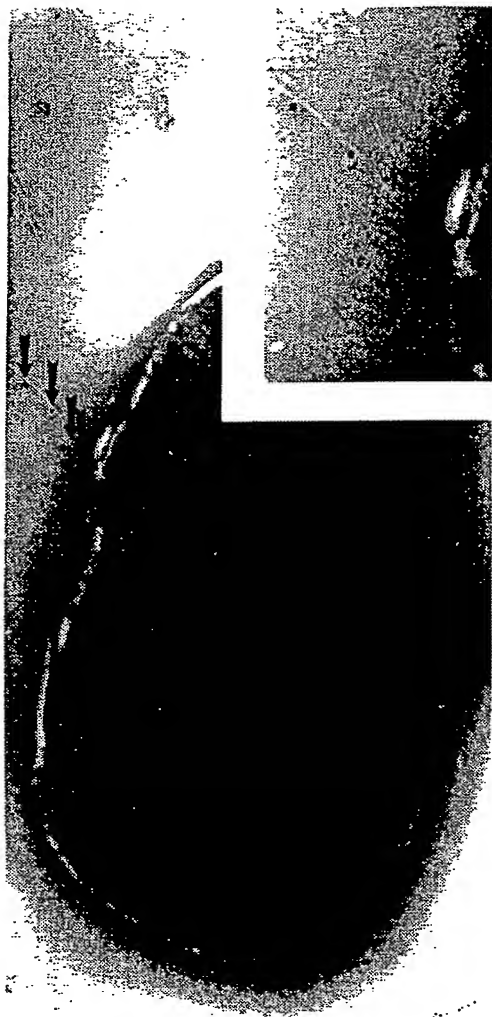GGTACCGGCTCGAGCAGTCAAGATCTCAATTCGCCGGCAGCT

FIG. 6

FIG. 7

FIG. 8

FIG. 9A-2

FIG. 9A-1

FIG. 9B-2

FIG. 9B-1

FIG. 10

FIG. 11

CDRI   CDRII   CDRIII   CH₁

FIG.   12

13/14
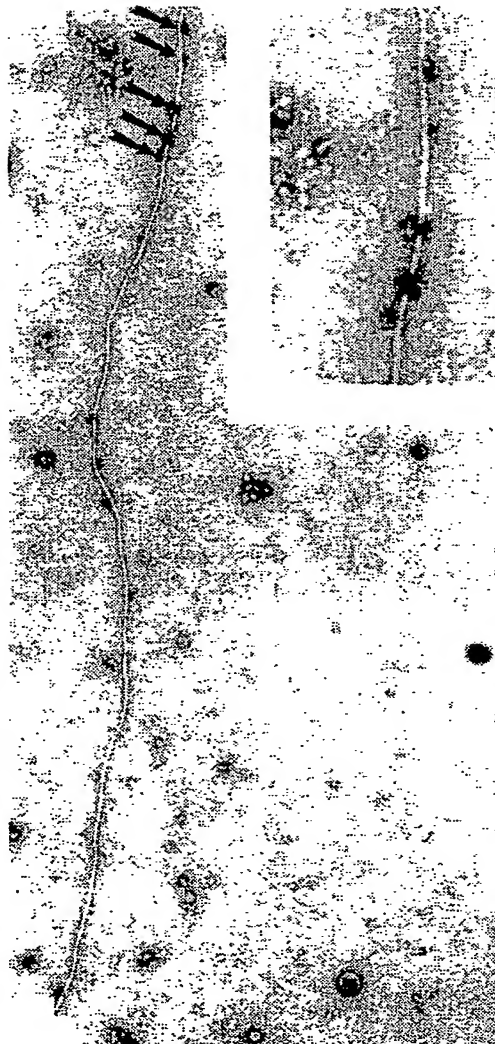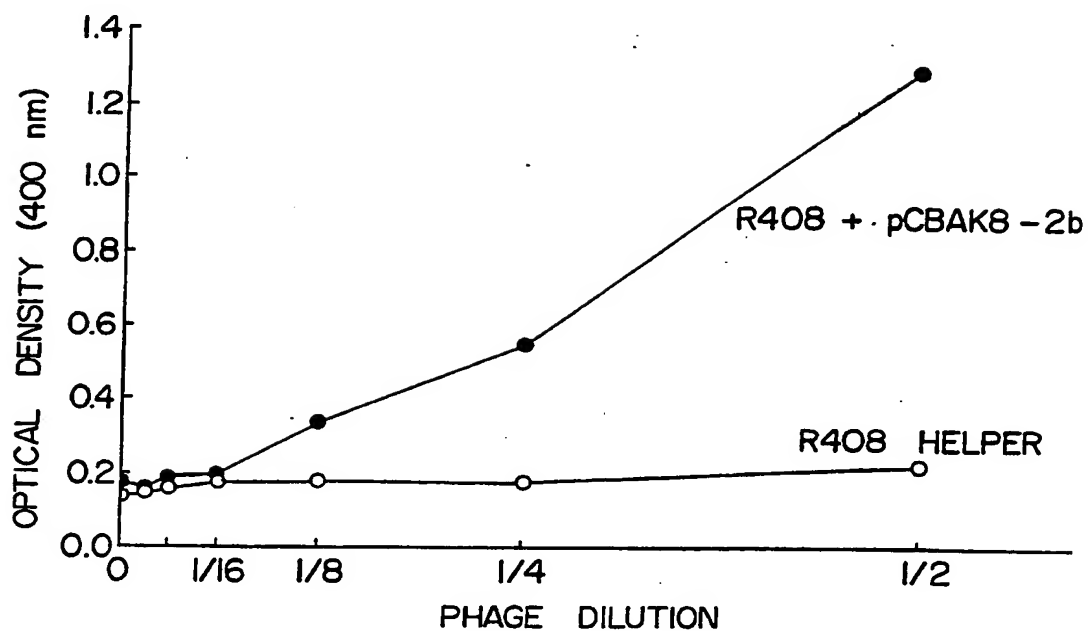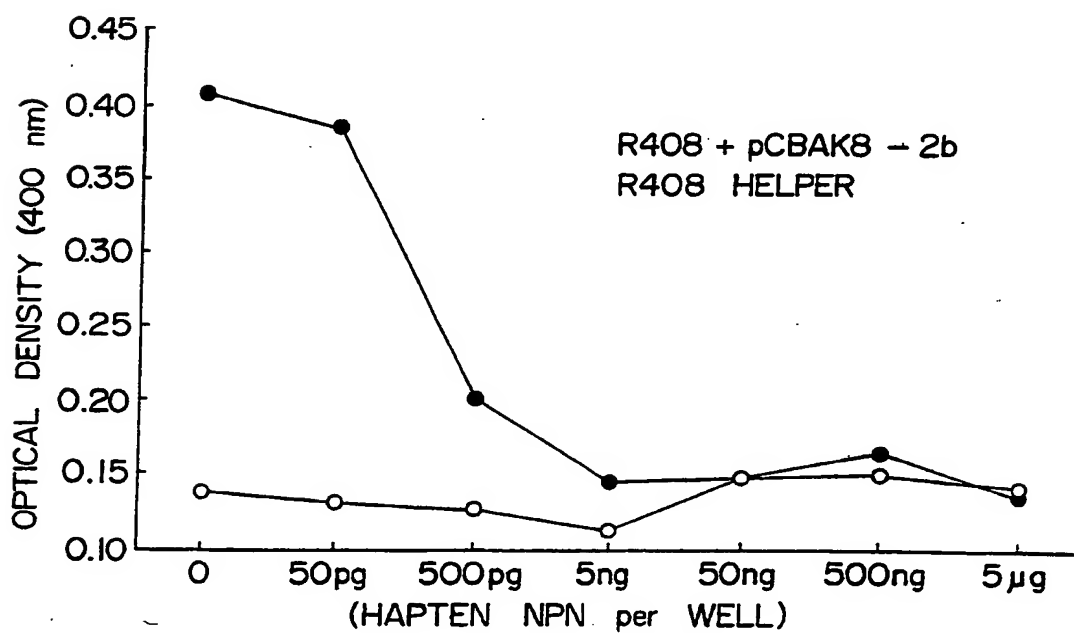
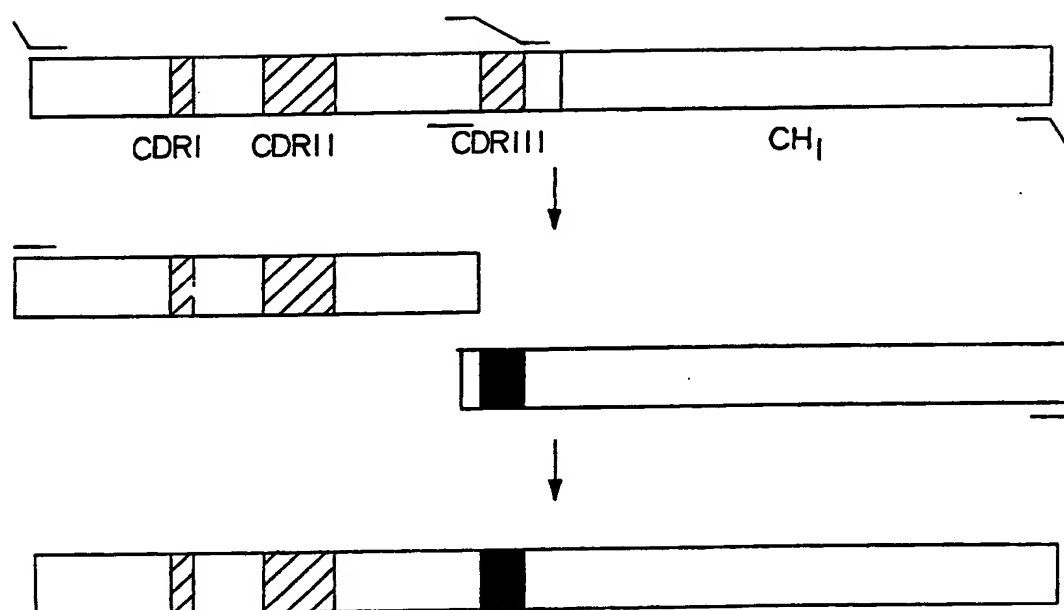| Clone	HCDR3 Sequence																K_d[F1] M	K_d[F1-BSA]* M	SEQ ID NO
	G	D	F	W	T	G	Y	S	D	Y	K	Y	A	M	D	V			
7E	G	N	R	M	R	G	L	R	S	R	P	V	M	M	D	L	$<10^{-5}$	$<10^{-5}$	130
F32	G	N	R	M	R	G	L	R	S	R	P	V	M	M	D	L			131
F34	G	N	R	M	R	G	L	R	S	R	P	V	M	M	D	L	6.0 X 10^{-7}	1 X 10^{-7}	132
F37	G	N	R	M	R	G	L	R	S	R	P	V	M	M	D	L			133
F1	G	V	N	L	L	R	V	N	S	S	R	P	H	L	D	M			134
F7	G	V	N	L	L	R	V	N	S	S	R	P	H	L	D	M			135
F9	G	V	N	L	L	R	V	N	S	S	R	P	H	L	D	M	1.4 X 10^{-7}	2 X 10^{-8}	136
F22	G	V	N	L	L	R	V	N	S	S	R	P	H	L	D	M			137
F25	G	V	N	L	L	R	V	N	S	S	R	P	H	L	D	M			138
F39	G	V	N	L	L	R	V	N	S	R	P	H	L	D	M	M			139
F31	G	G	P	V	S	R	A	L	R	R	Y	A	G	W	D	L	1.3 X 10^{-6}	3 X 10^{-8}	140
FBSA-8	V	A	S	Q	V	P	Q	R	A	K	R	P	M	F	D	D		5 X 10^{-8}	141
FBSA-11	F	L	A	F	R	L	R	W	P	L	J	R	A	G	N	S		1 X 10^{-7}	142
FBSA-13	G	L	P	G	H	G	R	S	P	F	T	G	P	P	S	Q		8 X 10^{-8}	143
FBSA-20	L	R	G	W	P	P	Y	R	G	G	Y	G	S	R	Q	R		1 X 10^{-7}	144
FBSA-30	S	H	R	T	S	W	Q	R	T	V	Y	R	Y	S	R	A			145
FBSA-48	L	R	K	P	Q	R	S	G	R	F	G	N	S	N	A	L	1.6 X 10^{-6}		146
FBSA-55	S	A	R	K	F	R	W	S	R	P	A	F	P	T	T	L			147

STARTING CLONE
FLUORESCEIN ELUTED CLONES
ACID ELUTED CLONES

FIG. 13

Clone	Light Chain CDR3 Corresponding to Kabat Positions 92-96	SEQ ID NO
Starting Clone		
7E	Gly Ser Ser Leu Trp	148
Fluorescein Eluted Clones		
p2	Thr Arg Pro Gly Val	149
p21	Thr Arg Pro Gly Val	149
p23	Thr Arg Pro Gly Val	149
p28	Thr Arg Pro Gly Val	149
p19	Thr Arg Pro Gly Val	149
p15	Ser Phe Lys Asn Trp	150
p11	Gly Tyr Arg Lys Trp	151

FIG. 14

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 7/01
US CL :435/235.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/235.1, 320.1, 172.3, 7.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nature, Vol. 348, issued 06 December 1990, J. McCafferty et al., "Phage antibodies: filamentous phage displaying antibody variable domains", pages 552-554, entire document.	1-3
Y	Science, Vol. 246, issued 08 December 1989, W. D. Huse et al., "Generation of a Large Combinatorial Library of the Immunoglobulin Repertoire in Phage Lambda", pages 1275-1281, entire document.	1-3
Y	Molekularnaya Biologiya, Vol. 24, No. 2, issued March 1990, A. A. Il'ichev et al., "M13 FILAMENTOUS BACTERIOPHAGE IN PROTEIN ENGINEERING", pages 530-535, entire document.	1-3

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 September 1992	28 SEP 1992

Name and mailing address of the ISA/	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	JAMES KETTER
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-1169

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

.ernational application No.

PCT/US92/03091

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
   Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any addtional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention frist mentioned in the claims; it is covered by claims Nos.:
   1-3

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.
   ☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)*

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

I: Claims 1-3 are drawn to filamentous phage particles.

II: Claims 4-13 are drawn to heterodimeric receptor molecules.

III: Claims 14-28 are drawn to expression vectors.

IV: Claims 29-31 are drawn to ligand binding receptor   fusion   polypeptides.

V: Claims 32-34 are drawn to libraries of filamentous phage.

VI: Claims 35-41 are drawn to oligonucleotide primers.

VII: Claims 42-48 are drawn to methods of inducing mutagenesis   utilizing the oligonucleotide primers of claims 35-41.

VIII: Claims 49-51 are drawn to a method of producing a DNA   library.

IX: Claim 52 is drawn to the DNA library produced using the   methods of claims 49-51.

X: Claims 53-59 are drawn to a method of increasing the   diversity of a library of filamentous phage.

XI: Claims 60-63 are drawn to a method of maturing the affinity   of a receptor expressed on a filamentous phage.

XII: Claims 64-67 are drawn to a method of detecting a   ligand   utilizing a heterodimeric receptor.

The claims of Group I are drawn to a product and as there are no claims to a method of making or a method of use of said first product, there are no other claims in this Group. The claims of Groups II-XII are drawn to distinct products and methods of use of said distinct products. PCT Rules 13.1 and 13.2 do not provide for multiple distinct products or their methods of use within a single general inventive concept. Note also 37 CFR § 1.475.

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